REQUIREMENT OF MYOD FOR MYOGENIC LINEAGE MAINTENANCE AND REGULATION OF SKELETAL MUSCLE TERMINAL DIFFERENTIATION BY THE MAPK SIGNALING PATHWAY

By

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ABSTRACT

The myogenic regulatory factors (MRFs) are crucial for the determination and terminal differentiation of the skeletal muscle lineage. Gene targeting experiments have demonstrated that one of *MyoD* or *Myf5* is required for establishing the myogenic lineage *in vivo*. To further understand the role of MyoD in lineage acquisition and differentiation, fibroblast cell lines lacking functional copies of *MyoD* and *Myf5* were generated and analyzed. The data shows that myogenin and MRF4 are capable of supporting terminal differentiation while at least one of MyoD or Myf5 is required for maintenance of myoblast identity. This represents the first direct evidence that maintenance of the myogenic lineage absolutely requires MyoD or Myf5. While expression of the MRFs is necessary for myogenesis, several extracellular growth factors repress their function, thereby maintaining myoblasts in a proliferative state. Growth factor stimulation leads to the activation of several intracellular signal transduction pathways. To understand the role of the mitogen-activated protein kinase (MAPK) pathway in regulating MRF function, experiments were performed to specifically address the effects of the MAPK signaling intermediate, MEK1. The data clearly shows that transcriptional activity of the MRFs is repressed when the MAPK pathway is activated. Repression of MyoD function occurs in the absence of direct MAPK phosphorylation, alterations in MyoD stability or subcellular localization. Remarkably, activated MEK1 localizes to the nucleus and binds directly to a transcriptional complex containing MyoD and its dimerization partner HEB. This data represents the first description of how the MAPK pathway controls myogenesis without suppressing the myoblast lineage. Data also revealed a critical cell cycle-specific loss of HEB as an additional regulatory mechanism for controlling myogenesis.

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"Success is on the far side of failure."- T.J. Watson, Founder of IBM

"The significant problems we face cannot be solved at the same level of thinking we were at when we created them."- Albert Einstein

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LIST OF ABBREVIATIONS

Act	activated (MEK1)
AP-1	activator protein-1
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cdk	cyclin-dependent kinase
CT	carboxyl terminal
DBD	DNA binding domain
DKO	double knock-out
DM	differentiation medium
DMEM	Dulbecco's modified eagle medium
DML	dorsal medial lip
DMSO	dimethylsulfoxide
DN	dominant negative (MEK1)
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine-tetraacetic acid
ER	estrogen receptor
ERK	extracellularly-regulated kinase
ES	embryonic stem
FBS	fetal bovine serum
FGF	fibroblast growth factor
FL	full-length
G1-phase	growth phase-1 of cell cycle
G2-phase	growth phase-2 of cell cycle
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein

GM	growth medium
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	haemagglutin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HEPES	(N-2-hyroxyethyl-piperazine-N'-2-ethanesulfonic acid) sodium salt
HGF/SF	hepatocyte growth factor/scatter factor
HRP	horseradish peroxidase
HS	horse serum
HU	hydroxyurea
IGF	insulin-like growth factor
IP	immunoprecipitation
JNK	jun amino-terminal kinase
K.O.	knock-out
lacZ	bacterial β-galactosidase
LTR	long terminal repeat
MADS	MCM1, agamous, deficiens and serum response factor
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MD	MyoD
MEF2	myocyte enhancer factor-2
MEFs	mouse embryonic fibroblasts
MEK	MAP/ERK kinase
MEKK	MEK kinase
MKK	mitogen-activated protein kinase kinase
MKKK	mitogen-activated protein kinase kinase kinase
MKP-1	MAPK phosphatase-1
MLC	myosin light chain
MNF	myocyte nuclear factor

mpcs	myogenic progenitor cells
MPF	mitosis-promoting factor
M-phase	mitosis phase of cell cycle
MRFs	myogenic regulatory factors
MSV	moloney sarcoma virus
MyHC	myosin heavy chain
NES	nuclear export signal
NFDM	non-fat dry milk
NLS	nuclear localization signal
NT	amino terminal
p.c.	post coitum
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platlet derived growth factor
p-ERK	phospho-ERK
PGK	phosphogycerate kinase
PI3'K	phosphatidylinosital 3'-kinase
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethyl sulfonyl fluoride
p-MEK	phospho-MEK
pRb (RB)	retinoblastoma protein
pTyr	phosphotyrosine
RNA	ribonucleic acid
rt	room temperature
RTK	receptor tyrosine kinase
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SH2	src homology 2
SH3	src homology 3
Shh	sonic hedgehog
S-phase	DNA synthesis phase of the cell cycle
SRE	serum response element
SRF	serum response factor
TAD	transactivation domain
TCF	ternary complex factor

TGF- β	transforming growth factor beta	UAS	upstream activating sequence
TLC	thin layer chromatography	UV	ultraviolet
TNFα	tumor necrosis factor alpha	VLL	ventral lateral lip
TPA	12-*O*-tetradecanoyl phorbol 13-acetate	wt	wild-type (MEK1)

CHAPTER 1

INTRODUCTION

Skeletal muscle of the mammalian adult arises during development from discrete structures located along the rostral-caudal axis called somites. Distinct molecular markers exist that permit detailed analyses of myogenic determination, myoblast proliferation and terminal differentiation. The myogenic regulatory factors (MRFs) are vital for determination and maintenance of the skeletal muscle lineage. During development, the induction of *MyoD* and *Myf5* expression defines the origin of myogenic progenitor cells (mpcs) that are responsible for forming distinct muscle groups of the adult organism. Gene targeting and transgenic mice have provided insight into the genetic relationships among the myogenic regulatory factor family and with molecules expressed within presumptive myogenic lineages. Interesting new insights have been uncovered explaining the molecular mechanisms that govern both proliferation and terminal differentiation. A number of signaling pathways have been shown to regulate myogenesis during development and regeneration of damaged tissue in the adult. These pathways regulate cell cycle progression, protein-protein interactions and transcriptional activity of the myogenic factors. However, questions persist regarding the precise molecular details involved with the nature of how several peptide growth factors repress the myogenic program. Of particular interest is the underlying mechanism of MRF transcriptional regulation while permitting myoblasts to maintain their myogenic identity.

1.1 DETERMINATION AND DEVELOPMENT OF THE MYOGENIC LINEAGE

1.1.1 Somitogenesis: Formation of Epaxial Versus Hypaxial Musculature

The formation of somite pairs on either side of the neural tube marks a crucial event during vertebrate development (for review see Pownall *et al.*, 2002). Somites form in a rostral to caudal direction and epithelization begins about day 7.5 postcoitum (p.c.) in the mouse. As development proceeds, somites become subdivided into the ventral sclerotome and dorsal dermomyotomal domains. Sclerotomal cells give rise to the vertebrae and ribs whereas the dermomyotome gives rise to the dermis of the back and the adult skeletal musculature of the trunk (for review see Dale and Pourquié, 2000; Brent and Tabin, 2002). Quail-chick somite grafts indicate that medial and lateral portions of somite are patterned by secreted factors from surrounding tissues and give rise to two distinct populations of myoblasts (Aoyama and Asamoto, 1988; Ordahl and LeDouarin, 1992; Hauschka, 1994). Cells located within the medial portion of the somite give rise to the muscles of the deep back, or epaxial muscles, whereas the lateral portion develops into the muscles of the body wall and limbs, or hypaxial muscles (Ordahl and LeDouarin, 1992).

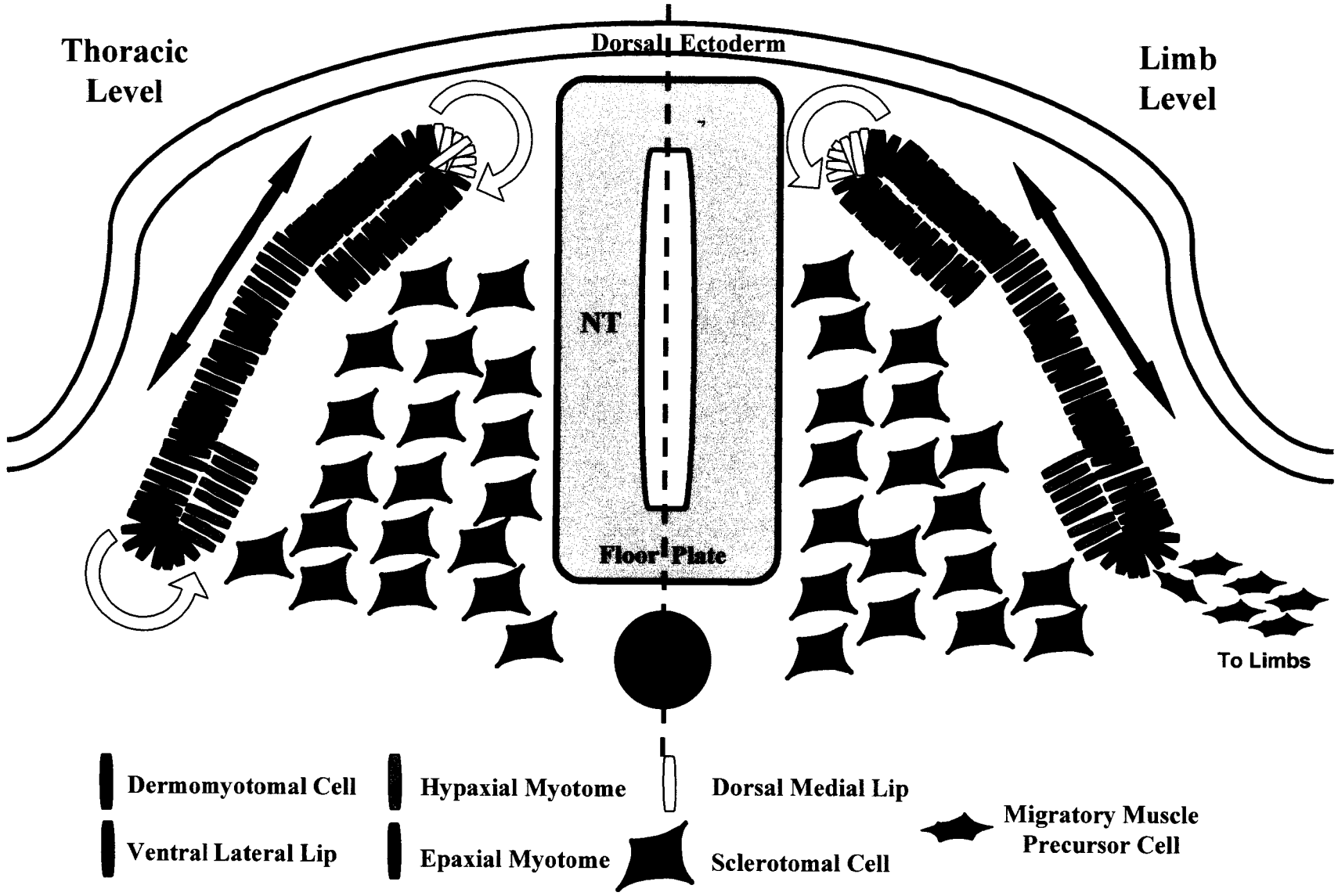
In birds, myotome formation occurs in sequential stages (for review see Kalcheim *et al.*, 1999; Pownall *et al.*, 2002). Initially, cells in the dorsomedial lip (DML) extend beneath the dermomyotome, exit the cell cycle, elongate and terminally differentiate (Denetclaw *et al.*, 1997; Kahane *et al.*, 1998a). These pioneer cells mark the first appearance of the myotome and are followed shortly after formation by a second wave of cells migrating from the rostral and caudal portions of the somite (Kahane *et al.*, 1998b).

The cells of the second wave originate in the DML and are dependent upon migration to enter the myotome from the correct position (Kahane *et al.*, 1998b; Denetclaw and Ordahl, 2000). Myotomal development from cells originating in the DML represents the epaxial portion of the myotome. A similar series of events occurs at the ventral lateral lip (VLL) of the dermomyotome leading to the formation of the non-migratory hypaxial portion of the myotome (Denetclaw and Ordahl, 2000; Cinnamon *et al.*, 1999). Subsequent expansion of the myotome occurs from the more superficial to deep regions of the myotome (Denetclaw and Ordahl, 2000).

At the limb level, events at the VLL occur differently (Figure 1.1). Cells of the VLL undergo an epithelial to mesenchymal transition, delaminate from the dermomyotome and migrate to regions of presumptive muscle development in the limbs (for review see Ordahl and Williams, 1998). Limb muscle formation occurs in temporally distinct waves involving at least two populations of cells that give rise to primary and secondary myotubes (Hauschka, 1994). *In vitro* characterization has shown that these two cell populations are distinguishable on the basis of clonal morphology and media requirements (White *et al.*, 1975; Seed and Hauschka, 1975; Seed and Hauschka, 1984). Moreover, the myosin heavy chain isoforms expressed by early and late cells differ, suggesting primary myoblasts are destined to give rise to slow muscle fibers whereas secondary myoblasts give rise to fast muscle fibers (for review see Stockdale, 1992). *In vivo* analyses of somite and limb grafts suggest that these early and late populations do indeed give rise to slow and fast fibers, respectively (Van Swearingen and Lance-Jones, 1995). Although injection of embryonic myoblasts into limbs of developing birds suggested that their lineage is maintained (DiMario *et al.*, 1993;

Figure 1.1: Somitic Origin of the Trunk Musculature.

The left side depicts events that occur in somites at the thoracic level and the right side shows events at limb-level somites. Dermomyotomal expansion leads to the extension of cells from the dorsomedial lip (DML) to a position beneath the dermomyotome. This marks the formation of the epaxial myotome which can be identified by *Myf5* expression. A similar extension occurs at the ventrolateral lip (VLL) forming the hypaxial myotome. The cells of the hypaxial myotome predominantly express *MyoD*. At the limb level, cells of the VLL delaminate and migrate to the developing limbs. These cells are *Pax3*, *Lbx1*, *c-Met* and *Msx1* positive. Upon arrival, these cells donwregulate Pax3 and initiate expression of the myogenic regulatory factors, in particular MyoD. Formation of the body wall musculature occurs via a continued ventral expansion of the myotome. It should be noted that the first appearance of the epaxial myotome occurs at day 8.5 whereas the first appearance of the hypaxial myotome is day 9.5. NT=neural tube; NC=notochord. (modified from Perry and Rudnicki, 2000).


Thoracic Level
Dorsal Ectoderm
Limb Level
NT
Floor Plate
To Limbs
Dermomyotomal Cell
Ventral Lateral Lip
Hypaxial Myotome
Epaxial Myotome
Dorsal Medial Lip
Sclerotomal Cell
Migratory Muscle Precursor Cell

DiMario and Stockdale, 1995), experiments in adults support a model in which environmental cues, such as innervation, play a substantial role in determining fiber-type potential (Hughes and Blau, 1992; Pin and Merrifield, 1997).

A third wave of migration, which represents the adult satellite cells, can be detected during the midfetal gestational stage in birds (Feldman and Stockdale, 1992). These cells appear to be of somitic origin, and are responsible for the majority of postnatal skeletal muscle growth (for review see Seale and Rudnicki, 2000; Goldring *et al.*, 2002). *In vitro*, these cells are phenotypically distinguished from primary and secondary myoblast populations (Feldman and Stockdale, 1991; Hartley *et al.*, 1992). Interestingly, analysis of clonal cultures from adult avian muscle suggests that satellite cells express a phenotype that is consistent with their fiber-type origin, although continued passaging of these cells indicates phenotypic plasticity (Feldman and Stockdale, 1991). Taken together, development of vertebrate trunk musculature involves multiple cell lineages that arise from spatially distinct regions of the somite. The myogenic regulatory factors (MRFs) are critical for the appropriate determination, development and maintenance of these skeletal muscle lineages.

1.1.2 Satellite Cell Origin

Early experiments using quail-chick chimeras suggested that satellite cells are somitically derived. These cells enter the limbs of mouse embryos at about day 17.5 p.c. (Hartley *et al.*, 1992; De Angelis *et al.*, 1999). More recent analyses examining satellite cell origin suggest that at least some of this cell population may actually arise in the dorsal aorta of embryonic mice (De Angelis *et al.*, 1999). Cultured cells isolated from the dorsal aorta coexpress skeletal muscle-specific and endothelial markers, similar to

adult satellite cells. Moreover, these cells are able to contribute to regenerating muscle (De Angelis *et al.*, 1999). Although *splotch* and *c-Met* null mice do not have cells migrate into the limb during development, cells isolated from the limbs of these embryos are myogenic, supporting the notion that at least some satellite cells originate from the vascular system (De Angelis *et al.*, 1999).

Important insight into the developmental and renewal aspects of the adult satellite cell compartment has come from analysis of mice lacking the paired-box transcription factor *Pax7* (Seale *et al.*, 2000). Close examination of *Pax7* null mice demonstrate that they lack essentially all satellite cells while having seemingly normal muscle at birth. *In situ* hybridizataion indicates *Pax7* is expressed in satellite cells of normal animals. Isolation of muscle-derived stem cells from *Pax7-/-* mice show that these cells are incapable of adopting a myogenic fate upon culturing compared to wild-type mice. This suggests the loss of Pax7 leads to an inability of cells to adopt a myogenic fate, implicating *Pax7* in a developmental program required for satellite cell specification (Seale *et al.*, 2000). However, other reports suggest that the origin of satellite cells is likely from multiple sources and not a single population of stem cells (reviewed in Goldring *et al.*, 2002). Further research is required to clarify the origins of satellite cells and the molecules involved in activating the myogenic program during post-natal development and adult regeneration.

1.1.3 The Myogenic Regulatory Factors (MRFs)

The original cloning of *MyoD* and demonstration that it represents a master regulatory gene for the determination of skeletal muscle, ushered in a new era of research in skeletal myogenesis (Davis *et al.*, 1987). This discovery lead to the cloning of three

other factors namely *Myf5* (Braun *et al.*, 1989a), *myogenin* (Wright *et al.*, 1989; Edmondson and Olson, 1989), and *MRF4/Myf-6/Herculin* (Rhodes and Konieczny, 1989; Braun *et al.*, 1990; Miner and Wold, 1990). In all cases, overexpresison of these factors converts non-muscle cells to the myogenic lineage, demonstrating their role in myogenic lineage determination and differentiation (Weintraub *et al.*, 1989; Choi *et al.*, 1990). Furthermore, the ability of each factor to initiate the expression of one or more of the other three suggests they form a cross-regulatory loop (Braun *et al.*, 1989b).

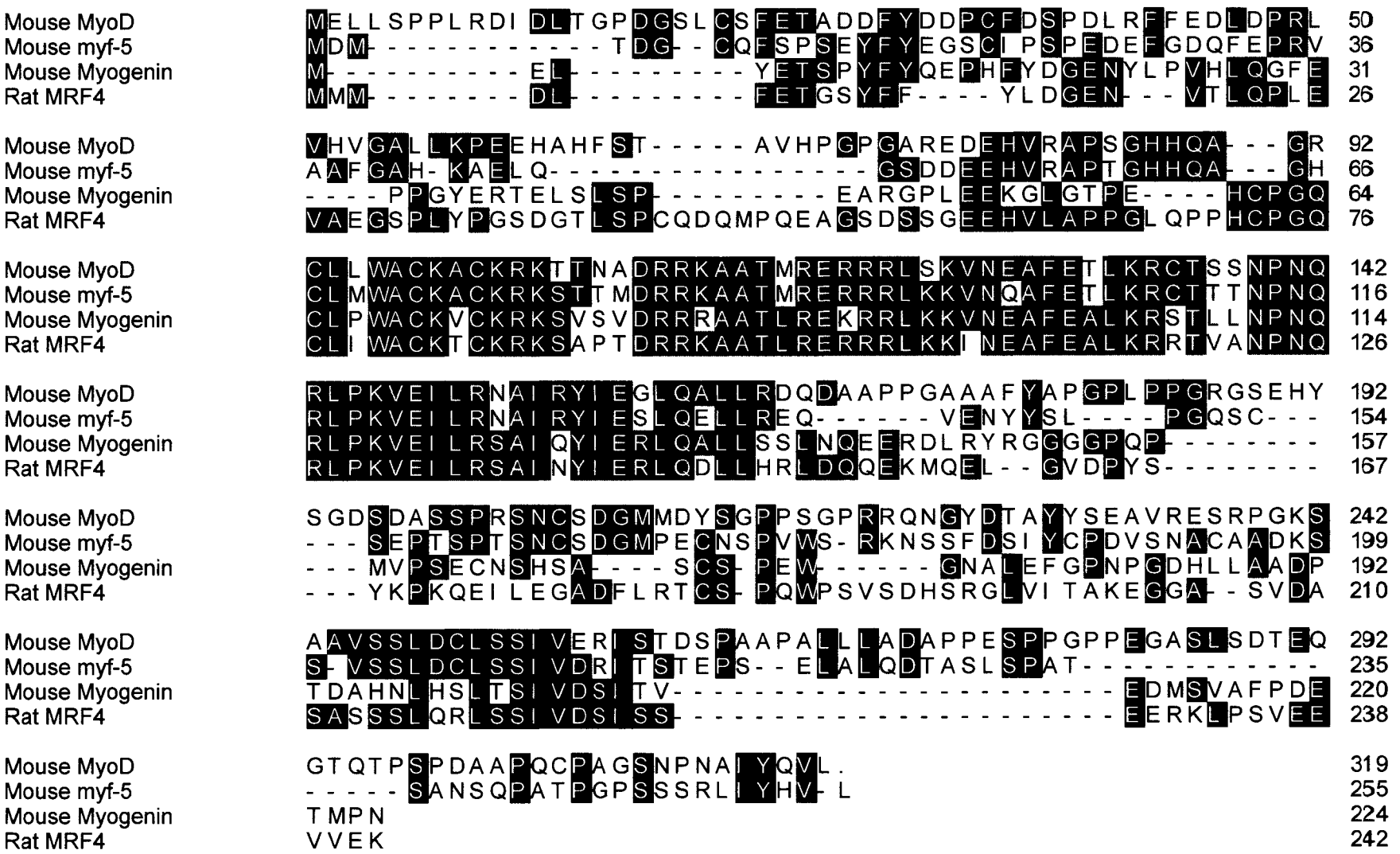
The MRFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors. The HLH domain is responsible for the dimerization of these factors with the ubiquitously expressed E-proteins, such as E12, E47, HEB, and ITF, and the basic domain is responsible for DNA binding (Murre *et al.*, 1989a; Lassar *et al.*, 1989; Lassar *et al.*, 1991). Heterodimers bind to the consensus E-box (CANNTG) DNA sequence motif found in the promoters of many muscle specific genes (Lassar *et al.*, 1989; Murre *et al.*, 1989b; Blackwell and Weintraub, 1990). The bHLH domains of the MRFs are highly homologous while the amino and carboxyl terminals show limited homology (Figure 1.2). Structurally, the MRFs contain several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, DNA binding, nuclear localization and heterodimerization (Tapscott *et al.*, 1988; Weintraub *et al.*, 1991; Vandromme *et al.*, 1995; Gerber *et al.*, 1997; Schwarz *et al.*, 1992).

1.1.4 Developmental Expression of the Myogenic Regulatory Factors

During development the MRFs are expressed in a highly regulated spatial and temporal fashion (for review see Pownall *et al.*, 2002). *In situ* hybridization has shown that MRF expression occurs in slightly different patterns in epaxial versus hypaxial

Figure 1.2: Amino Acid Alignment of the Myogenic Regulatory Factors.

Alignment of the amino acid sequences of the four myogenic regulatory factors. Black boxes show areas where at least two of the factors have a conserved residue. The yellow highlighted region represents the basic helix-loop-helix domain (MyoD amino acids 110-165) of the molecules. It can be seen that the amino and carboxyl-terminal regions show limited homology. This suggests that each MRF has specific and separable functions.


Mouse MyoD MELLSPPLRDI DLTGPDGSLCSFETADDFYDDPCFDSPDLRFFEDLDPRL 50
Mouse myf-5 MDM----------TDG--CQFSPSEYFYEGSCIPSPEDEFGDQFEPRV 36
Mouse Myogenin M----------EL----------YETSPYFYQEPHFYDGENYLPVHLQGFE 31
Rat MRF4 MMM--------DL----------FETGSYFF----YLDGEN---VTLQPLE 26
Mouse MyoD VHVGALLKPEEHAHFST-----AVHPGPGAREDEHVRAPSGHHQA---GR 92
Mouse myf-5 AAFGAH-KAELQ----------GSDDEEHVRAPTGHHQA---GH 66
Mouse Myogenin ----PPGYERTELSLSP---------EARGPLEEKGLGTPE----HCPGQ 64
Rat MRF4 VAEGSPLYPGSDGTLSPCQDQMPQEAGSDSSGEEHVLAPPGLQPPHCPGQ 76
Mouse MyoD CLLWACKACKRKTTNADRRKAATMRERRRLSKVNEAFETLKRCTSSNPNQ 142
Mouse myf-5 CLMWACKACKRKSTTMDRRKAATMRERRRLKKVNQAFETLKRCTTTNPNQ 116
Mouse Myogenin CLPWACKVCKRKSVSVDRRRAATLREKRRLKKVNEAFEALKRSTLLNPNQ 114
Rat MRF4 CLIWACKTCKRKSAPTDRRKAATLRERRRLKKINEAFEALKRRTVANPNQ 126
Mouse MyoD RLPKVEILRNAIRYIEGLQALLRDQDAAPPGAAAFYAPGPLPPGRGSEHY 192
Mouse myf-5 RLPKVEILRNAIRYIESLQELLREQ------VENYYSL----PGQSC--- 154
Mouse Myogenin RLPKVEILRSAIQYIERLQALLSSLNQEERDLRYRGGGGPQP-------- 157
Rat MRF4 RLPKVEILRSAINYIERLQDLLHRLDQQEKMQEL--GVDPYS-------- 167
Mouse MyoD SGDSDASSPRSNCSDGMMDYSGPPSGPRRQNGYDTAYYSEAVRESRPGKS 242
Mouse myf-5 ---SEPTSPTSNCSDGMPECNSPVWS-RKNSSFDSIYCPDVSNACAADKS 199
Mouse Myogenin ---MVPSECNSHSA----SCS-PEW------GNALEFGPNPGDHLLAADP 192
Rat MRF4 ---YKPKQEILEGADFLRTCS-PQWPSVSDHSRGLVITAKEGGA--SVDA 210
Mouse MyoD AAVSSLDCLSSIVERISTDSPAAPALLLADAPPESPPGPPEGASLSDTEQ 292
Mouse myf-5 S-VSSLDCLSSIVDRITSTEPS--ELALQDTASLSPAT---------- 235
Mouse Myogenin TDAHNLHSLTSIVDSITV--------------------EDMSVAFPDE 220
Rat MRF4 SASSSLQRLSSIVDSISS--------------------EERKLPSVEE 238
Mouse MyoD GTQTPSPDAAPQCPAGSNPNAIYQVL. 319
Mouse myf-5 -----SANSQPATPGPSSSRLIYHV-L 255
Mouse Myogenin TMPN 224
Rat MRF4 VVEK 242

muscle. *Myf5* expression is detected in the dorsomedial portion of the somite at day 8 p.c. and at day 9.5 in the lateral, or hypaxial domain of the somite (Ott *et al.*, 1991; Tajbakhsh *et al.*, 1996a). *Myogenin* is first detected at day 8.5 p.c. and remains detectable throughout fetal development (Sassoon *et al.*, 1989). *MRF4* expression is detected transiently between days 10 and 11 and then reexpressed from day 16 onward to become the predominant MRF expressed in adult muscle (Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Hannon *et al.*, 1992). *MyoD* expression is first detected approximately day 9.75 in the hypaxial somitic domain and continues to be expressed throughout development (Bober *et al.*, 1991; Faerman *et al.*, 1995). In the limb bud, the temporal appearance of these factors differs. Although *Myf5* expression is again detected first, it is followed very quickly by *MyoD* and *myogenin* which are detected from day 10.5 onward (Ott *et al.*, 1991; Sassoon *et al.*, 1989). Unlike observations in the somite, *MRF4* is not transiently expressed during limb development but is first detected at day 16 and becomes the predominant MRF expressed in the adult (Bober *et al.*, 1991; Hinterberger *et al.*, 1991).

1.1.5 Lessons From Gene Targeting

Targeted inactivation of the MRFs has provided a great deal of insight into the nature of lineage determination, lineage maintenance and MRF genetic hierarchies. Mice lacking a functional copy of *MyoD* are viable without any obvious defects in skeletal muscle (Rudnicki *et al.*, 1992). Initial gene targeting experiments of the Myf5 locus revealed essentially normal muscle but the mice die perinatally due to a severe rib development defect (Braun *et al.*, 1992; Tajbakhsh *et al.*, 1996a). These mice do not show changes in the expression pattern of the other MRFs but do demonstrate a delay in

myotome development (Braun *et al.*, 1992). Later studies revealed that changes in the construction of the gene targeting vector yielded mice that were normal and fertile suggesting that a gene nearby the *Myf5* locus important for rib development is dysregulated (Kaul *et al.*, 2000). Mice lacking both *MyoD* and *Myf5* genes show a complete absence of myoblasts and muscle fibers. This demonstrates that at least one of these factors is required for determining the myogenic lineage during embryonic development and activation of *myogenin* and *MRF4* are dependent upon the preceding expression of MyoD and/or Myf5 (Rudnicki *et al.*, 1993; Kaul *et al.*, 2000).

Gene targeting of the *myogenin* locus provided the first indication of the importance of the MRFs during development. In accordance with the appearance of *myogenin* at the onset of differentiation, lack of myogenin leads to perinatal death due to a severe deficiency of differentiated muscle fibers in newborn mice (Hasty *et al.*, 1993; Nabeshima *et al.*, 1993). Areas of presumptive muscle development have normal numbers of myonuclei and these cells are capable of differentiation when cultured *in vitro* (Nabeshima *et al.*, 1993). Interestingly, closer examination of *myogenin* null mice indicates that primary muscle fiber formation is unaffected whereas there are defects in secondary fiber myogenesis (Venuti *et al.*, 1995).

Three laboratories inactivated the *MRF4* gene yielding a range of defective rib cage phenotypes (Braun and Arnold, 1995; Patapoutian *et al.*, 1995; Zhang *et al.*, 1995). The severity of the rib phenotype correlates with perturbations in *Myf5* expression, which lies approximately 6 kilobases away suggesting *cis*-regulatory elements (Olson *et al.*, 1996; Yoon *et al.*, 1997; Kaul *et al.*, 2000). The most severe rib defects are observed in mice that do not show any detectable expression of *Myf5* (Braun and Arnold, 1995).

Moderate perturbation of *Myf5* expression leads to alterations in myotomal muscle development and rib abnormalities (Patapoutian *et al.*, 1995). Mice with normal *Myf5* expression are born healthy and fertile with minor rib abnormalities and show a four-fold increase in *myogenin* expression (Zhang *et al.*, 1995; Rawls *et al.*, 1995). This suggests increases in myogenin levels are able to compensate for the lack of *MRF4*.

Together, the gene targeting experiments suggest a model in which MyoD and Myf5 act to determine the myoblast lineage whereas myogenin and MRF4 are important for differentiation and maintenance of the terminally differentiated state (Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995).

To further understand the functional relationships of the MRFs, mice lacking multiple MRFs or, mice in which the coding sequence of one MRF has been knocked-in to the locus of another, have been examined. Mice lacking functional copies of both *myogenin* and *MyoD*, *myogenin* and *Myf5*, *myogenin* and *MRF4* or, lack all but *Myf5* are phenotypically identical to *myogenin* null mice indicating that myogenin is genetically downstream of both *MyoD* and *Myf5* (Rawls *et al.*, 1995; Rawls *et al.*, 1998; Valdez *et al.*, 2000). Surprisingly, mice lacking *MRF4* and *MyoD* yield a phenotype similar to that of myogenin null mice (Rawls *et al.*, 1998). This indicates that *myogenin* can only compensate for the lack of *MRF4* in the presence of *MyoD* expression lending support to the hypothesis that distinct lineages are defined by *MyoD* and *Myf5* expression.

Substitution of the coding region of *myogenin* into the *Myf5* locus ($Myf5^{myg\text{-}ki}$) rescues the rib defect in a *Myf5* null background (Wang *et al.*, 1996). However, mice homozygous for $Myf5^{myg\text{-}ki}$ in a *MyoD* null background die perinatally due to reduced muscle formation (Wang and Jaenisch, 1997). Furthermore, $Myf5^{myg\text{-}ki}$ in a *myogenin* null

background are born with a *myogenin* null phenotype showing that the early expression of myogenin is unable to compensate at later time points of differentiation (Wang and Jaenisch, 1997). Using overexpression studies in *myogenin* -/- embryonic stem (ES) cells it has been shown that MyoD is unable to compensate for the lack of myogenin during terminal differentiation (Myer *et al.*, 2001), whereas MRF4 rescues the defect (Sumariwalla and Klein, 2001). While the data suggests that there is a critical threshold level of MRF expression required for proper muscle formation, it is clear there are non-redundant functions necessary for specific aspects of myogenesis (Valdez *et al.*, 2000; Myer *et al.*, 2001; Sumariwalla and Klein, 2001).

To obtain a greater understanding of how *MyoD* and *Myf5* serve to determine lineages within the developing myotome, our laboratory examined the expression patterns of two transgenes that drive the expression of the bacterial β-galactosidase (*lacZ*) gene under control of *MyoD* promoter elements. The upstream MD6.0-*lacZ* (6.0 kilobases of upstream MyoD promoter sequence) is detected in differentiated myocytes (Asakura *et al.*, 1995), whereas the 258/-2.5*lacZ* transgene (which has the 258 base pair – 20 kilobase core enhancer fused to 2.5 kilobases of the MyoD promoter) is detected in determined myoblasts (Goldhamer *et al.*, 1995).

Mice lacking *Myf5* demonstrate a 2.5 day delay in development of paraspinal, intercostal and limb muscles (Kablar *et al.*, 1997; Kablar *et al.*, 1998), confirming previous reports that delayed expression of *MyoD* in a *Myf5* null background marks the onset of muscle differentiation (Braun *et al.*, 1994). By contrast, mice lacking *MyoD* demonstrate normal epaxial muscle formation while hypaxial muscle development is delayed approximately 2 days (Kablar *et al.*, 1997; Kablar *et al.*, 1998). These results

provide strong evidence that epaxial musculature is dependent upon *Myf5* expression whereas *MyoD* is required for appropriate hypaxial muscle formation (Kablar and Rudnicki, 2000).

To examine whether the migratory hypaxial population of cells are affected in the absence of MRF expression, mice lacking both *MyoD* and *Myf5* were examined using the 258/-2.5*lacZ* transgene (Kablar *et al.*, 1999). Expression of *lacZ* is detected in both newly formed somites and limb buds. This pattern of staining demonstrates that in the absence of MRF expression, activation of the –20 kilobase enhancer of *MyoD* occurs and cell migration to the limbs is unaffected (Kablar *et al.*, 1999). Interestingly, many *lacZ* positive cells in both the somitic and limb bud regions adopt non-myogenic fates suggesting these cells are multipotential (Kablar *et al.*, 1999). This confirms reports demonstrating that in the absence of *Myf5*, cells migrate abnormally and have an increased propensity to terminally differentiate along non-myogenic cell fates (Tajbakhsh *et al.*, 1996b). The importance of *Myf5* for certain myogenic lineages is strengthened by the fact that smooth muscle cells of the esophagus are delayed in their transdifferentiation to skeletal muscle in the absence of *Myf5* expression (Kablar *et al.*, 2000).

Taken together, the data obtained from transgenic mice clearly demonstrates that *MyoD* and *Myf5* are responsible for the determination of two distinct populations of muscle cells in the myotome. However, the precise mechanisms involved with initiating the expression of *MyoD* versus *Myf5* remains unclear.

1.2 REGULATION OF MYOGENESIS DURING DEVELOPMENT

1.2.1 Extracellular Cues Regulating Myogenic Determination

Several factors are expressed in axial and lateral regions of the developing embryo which are important for somite formation and the determination of cell lineages (for review see Pownall *et al.*, 2002). Axial structures, such as the neural tube and notochord, provide signals necessary for epaxial myogenic determination (Teillet and LeDouarin, 1983; Rong *et al.*, 1992; Pourquié *et al.*, 1993; Buffinger and Stockdale, 1994; Spence *et al.*, 1996; Pownall *et al.*, 1996). By contrast, the hypaxial myogenic lineage is dependent upon signals originating from the lateral plate mesoderm and dorsal ectoderm (Pourquie *et al.*, 1995; Pourquie *et al.*, 1996; Cossu *et al.*, 1996; Kenny-Mobbs and Thorogood, 1987; Fan and Tessier-Lavigne, 1994; Dietrich *et al.*, 1997). Factors secreted from these structures include sonic hedgehog (Shh), Wnts, transforming growth factor–beta (TGF-β)-like molecules, fibroblast growth factors (FGFs) and the bone morphogenic proteins (BMPs). All of these factors regulate myogenic determination and differentiation. However, there are differential effects observed between epaxial and hypaxial musculature.

Sonic hedgehog (Shh) is expressed from the notochord and neural floor plate and has been shown to positively regulate the formation and survival of the dorsal myotome, (Johnson *et al.*, 1994; Münsterberg *et al.*, 1995; Teillet *et al.*, 1998). Mice lacking *Shh* show reduced *Myf5* expression in the expaxial myotome (Chiang *et al.*, 1996; Borycki *et al.*, 1999), however, formation of the hypaxial myotome and MyoD expression is unaffected (Borycki *et al.*, 1999). In association with Shh, several Wnts have been shown to induce myogenesis and are thought to synergistically act with Shh (Münsterberg *et al.*, 1995; Stern *et al.*, 1995; Marcelle *et al.*, 1997). Mice lacking both *Wnt-1* and *Wnt-3a* are unable to from the medial dermomyotome but show normal

development of the lateral myotome (Ikeya and Takada, 1998). Interestingly, Wnt-1 induces *Myf5* expression whereas Wnt-7a, which is expressed in the lateral plate mesoderm, induces *Myo*D expression (Tajbakhsh *et al.*, 1998). These results confirm previous studies demonstrating that the neural tube induces *Myf5* expression while the dorsal ectoderm preferentially activates *MyoD* expression (Cossu *et al.*, 1996). Taken together, this data indicates the importance of Shh and Wnt signaling during development. Moreover, it confirms the hypothesis that epaxial and hypaxial musculature represent distinct lineages dependent on *Myf5* and *MyoD* expression, respectively.

The BMPs belong to the TGF-β family of secreted factors and information obtained thus far shows these factors negatively regulate myogenesis. In particular, BMP4 has been of some interest due to its high level of expression in the lateral plate mesoderm (Pourquie *et al.*, 1996). Experiments looking at the effects of BMPs on cells strongly suggest BMP concentration gradients are vital for cells to respond appropriately (Amthor *et al.*, 1998). Low BMP levels in the limb bud maintain migrating, *Pax3* expressing myogenic precursor cells in a proliferative state and repress myogenesis. By contrast, high BMP concentrations induce cell death (Amthor *et al.*, 1998). Important aspects of BMP signaling are the patterns of expression of BMPs and their inhibitors follistatin, noggin and chordin. Expression of the BMP antagonist, noggin, in the DML and lateral plate regulates the development of both medial and lateral myogenic lineages (Marcelle *et al.*, 1997; Hirsinger *et al.*, 1997; Reshef *et al.*, 1998). Indeed, ectopic expression of noggin in the lateral regions of the embryo represses *Pax3* expression, expands the *MyoD* expression domain, and induces myogenesis (Reshef *et al.*, 1998).

Several FGF and TGF-β family members have been identified. Treatment of cultured myoblasts with these factors suggests they act to stimulate proliferation and repress terminal differentiation. However, *in vivo* these molecules are important for the formation and terminal differentiation of the dorsal myotome (Stern *et al.*, 1997). Neutralizing antibodies to TGF-β or basic-FGF (bFGF) inhibit myotomal induction by axial structures. Exposure of segmental plate explants to a combination of TGF-β and bFGF induces myotome formation. TGF-β acts to specify the cells to the myogenic lineages whereas bFGF acts to promote proliferation and cell survival (Stern *et al.*, 1997). Other TGF-β and FGF molecules have been shown to play a role during regeneration and these will be discussed below (Section 1.3.1).

Cell-cell contact during development represents an important mechanism that contributes to the formation of distinct cell types. The transmembrane proteins of the *Notch-Delta/Jagged* signaling pathway are involved with cell contact signaling (Artavanis-Tsakonas *et al.*, 1999). Upon interaction of a Notch expressing cell with a Delta/Jagged expressing cell, the intracellular portion of Notch is cleaved, translocates to the nucleus and suppresses differentiation. Overexpression of the cytoplasmic portion of Notch represses myogenesis (Kopan *et al.*, 1994). During development, *Notch2* is expressed in cells of the DML, which lie juxtaposed to *Delta* expressing cells in the developing somite (Weinmaster *et al.*, 1992; Williams *et al.*, 1995). This suggests that Notch2 suppresses myogenic commitment prior to cells extending beneath the dermomyotome.

1.2.2 Genes Important For Myoblast Migration During Development

Migration of cells from the VLL to the developing limb buds is required for the formation of limb hypaxial musculature. The naturally occurring *splotch* mutant mouse does not develop limb musculature (Franz *et al.*, 1993). This is due to a loss-of-function mutation in the *Pax3* gene which is required for cells of the VLL to migrate (Goulding *et al.*, 1994; Bober *et al.*, 1994; Williams and Ordahl, 1994). It should be noted that although migration of muscle precursor cells is impaired, transplantation of these cells from the VLL to the limb bud shows they are capable of terminal differentiation (Daston *et al.*, 1996). Overexpression of Pax3 in cells represses myogenesis suggesting that it is involved with maintaining migrating myoblasts in an undifferentiated state (Amthor *et al.*, 1998). Indeed, upregulation of Pax3 occurs in cells exposed to BMP signals from the dorsal ectoderm and limb buds, thus permitting muscle precursor cells to migrate and proliferate prior to differentiation (Pourquie *et al.*, 1996; Amthor *et al.*, 1998; Amthor *et al.*, 1999).

Although cells that do not migrate in *splotch* mice are specified to the myogenic lineage, there is evidence that Pax3 is involved with determination of the myogenic lineage. Generation of mice lacking *Myf5* in a *splotch* background demonstrates a surprising genetic relationship between *Pax3*, *MyoD* and *Myf5* (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997). S*plotch* mice demonstrate normal myotomal development and activation of *MyoD*. However, *splotch* mice lacking *Myf5* do not form any musculature due to a lack of *MyoD* expression in the developing somite (Maroto *et al.*, 1997; Tajbakhsh *et al.*, 1997). Moreover, exposure of paraxial mesoderm explants to Pax3 can induce myogenic differentiation, supporting a role for Pax3 in activating *MyoD* in a *Myf5* independent pathway during somitogenesis (Maroto *et al.*, 1997).

The c-Met receptor tyrosine kinase and its cognate ligand hepatocyte growth factor/scatter factor (HGF/SF) are important for the migration of myogenic cells. Targeted disruption of the *c-Met* or *HGF/SF* genes leads to a similar phenotype as that observed in *splotch* mice (Bladt *et al.*, 1995; Maina *et al.*, 1996). Similar to *splotch* mice, there are not any defects in myotomal development. Moreover, although migratory cells of the VLL do not delaminate and migrate to the limbs, they are specified to the myogenic lineage, as observed in *splotch* mice (Bladt *et al.*, 1995; Dietrich *et al.*, 1999).

Lbx1 is a homeobox protein expressed in the VLL and in *Pax3* positive migrating cells. Targeted inactivation of *Lbx1* leads to a disruption of only a subset of forelimb muscles and complete ablation of hindlimb musculature (Schäfer and Braun, 1999; Brohmann *et al.*, 2000; Gross *et al.*, 2000). Specifically, forelimb extensor muscles are absent, implicating Lbx1 in the dorsoventral migration pattern of myogenic precursor cells during development (Schäfer and Braun, 1999; Brohmann *et al.*, 2000; Gross *et al.*, 2000). Interestingly, *Lbx1* expression is not detected in the trunk-level dermomyotomes of *splotch* mice suggesting that in certain regions of the developing embryo, Pax3 is involved with activation of Lbx1 expression (Mennerich *et al.*, 1998).

Msx1 is a homeodomain protein that demonstrates overlapping expression with *Pax3* and represses myogenesis *in vitro* (Woloshin *et al.*, 1995). Interestingly, Msx1 has recently been shown to be antagonistic to both *Pax3* and MRF expression. This regulation is mediated by direct interaction between Msx1 and Pax3, blocking Pax3 DNA binding, and is important for controlling the timing of myogenesis in the limb (Bendall *et al.*, 1999; Houzelstein *et al.*, 1999).

The molecules responsible for activating MRF expression during development are currently unknown. However, recent studies examining the expression and activity of Dach2, Eya, Six1 and Pax3 proteins have suggested one mechanism by which the MRFs may be activated in the myotome (Heanue *et al.*, 1999; Relaix and Buckingham, 1999). These four molecules are expressed in the dermomyotome, myotome and the migratory population of cells in the VLL (Relaix and Buckingham, 1999). Dach2 and Pax3 positively autoregulate the expression of each other and myogenesis is induced within the somite by expression of Dach2/Eya2 or Six1/Eya2 complexes (Heanue *et al.*, 1999). Although it is not known whether these transcription factor complexes activate the promoters of *MyoD* or *Myf5* directly, it is clear that these proteins are likely responsible for the ability of ectopic Pax3 expression to induce myogenesis in non-muscle tissue (Maroto *et al.*, 1997).

1.3 REGENERATION OF ADULT SKELETAL MUSCLE: SATELLITE CELLS

In adult muscle, approximately 5% of the myonuclei present in muscle fibers represent satellite cells (Bischoff, 1994). Normally, these cells are mitotically quiescent but can be induced to proliferate by stresses, such as physical trauma or weight-bearing (for review see Grounds, 1998; Seale and Rudnicki, 2000). Activated satellite cells undergo multiple rounds of cell division, exit the cell cycle and fuse onto the existing damaged fibers (Grounds, 1998). Several potential factors exist within the area of damage that may serve to activate satellite cells (Seale and Rudnicki, 2000). Single-cell RT-PCR (reverse-transcription polymerase chain reaction) experiments show that quiescent satellite cells do not express detectable levels of MRFs, but do express the Met receptor and the muscle cell adhesion molecule M-cadherin (Cornelison and Wold,

1997). Upon activation, cells express either *MyoD* or *Myf5*, but eventually express both prior to progression through the differentiation program (Cornelison and Wold, 1997). What is unclear is how the satellite cell compartment is renewed.

Insight into satellite cell renewal has come from experiments examining the role of the MRFs during regeneration. Although *MyoD* null mice are born without apparent defects in skeletal muscle, when these mice are interbred with the *mdx* mouse or, adult muscle is subjected to damage, muscle regeneration is severely impaired even though several cells are detected in the damaged area, these mice are unable to efficiently regenerate (Megeney *et al.*, 1996). *In vitro* analysis of cells isolated from adult *MyoD* null mice demonstrate that these cells are unable to progress through the normal differentiation program and are mitotically active under conditions that initiate terminal differentiation in wild-type control cells (Sabourin *et al.*, 1999; Yablonka-Reuveni *et al.*, 1999). Although *MyoD-/-* cells express high levels of *Myf5*, their ability to terminally differentiate is impaired (Sabourin *et al.*, 1999). Taken together, these results indicate that cells lacking *MyoD* may represent an intermediate phenotype between quiescent satellite cell and determined myogenic progenitor cell (mpc) (Sabourin *et al.*, 1999; Sabourin and Rudnicki, 2000). Moreover, the expression of *Myf5* alone is insufficient for differentiation, suggesting that renewal of the satellite cell compartment may be a function of Myf5 expression (Sabourin *et al.*, 1999; Sabourin and Rudnicki, 2000).

Other studies suggest the winged helix transcription factor MNF (myocyte nuclear factor) is essential for the maintenance of satellite cells. MNF expression is detected in quiescent satellite cells (Garry *et al.*, 1997). Two alternatively spliced isoforms can be detected with the beta isoform expressed in quiescent cells and the alpha isoform in

activated mpcs (Yang *et al.*, 1997; Garry *et al.*, 2000). Interestingly, mice lacking a functional copy of MNF show severe deficiencies in skeletal muscle regeneration and are unable to properly coordinate the expression of cell cycle and myogenic determination genes (Garry *et al.*, 2000). This suggests that MNF serves to properly activate genes responsible for determining mpcs and activating the myogenic program.

During regeneration, expansion of the mpc compartment is necessary for proper muscle repair to occur. FGF6 demonstrates a skeletal muscle-specific pattern of expression (deLapeyriere *et al.*, 1993; Coulier *et al.*, 1994; Pizette *et al.*, 1996). Mice lacking *FGF6* are born healthy and fertile with no developmental abnormalities in skeletal muscle (Floss *et al.*, 1997; Fiore *et al.*, 1997). However, these mice demonstrate a reduced capacity for regeneration after mechanical injury or when interbred with the *mdx* mouse (Floss *et al.*, 1997). Although FGF6 null mice have normal numbers of satellite cells, activation of the regeneration program yields fewer MyoD and myogenin positive cells and an increased deposition of collagen in sites of regeneration. This suggests that FGF6 represses differentiation and permits expansion of the satellite cell compartment during adult skeletal muscle regeneration (Floss *et al.*, 1997).

By contrast, targeted inactivation of the TGF-β family member, GDF8 or myostatin, results in mice with substantial increases in muscle mass (McPherron *et al.*, 1997). Both hyperplasia and hypertrophy are responsible for this increase in muscle mass. Unlike FGF6, GDF8 is a negative regulator of myoblast proliferation and differentiation and is involved with hypertrophic effects which is mediated by satellite cells (McPherron *et al.*, 1997).

Information from previous sections and above has demonstrated that members of the TGF- β family are responsible for repressing the determination of myogenic cells whereas FGF family members stimulate proliferation of myogenic cells. Of interest are recent studies showing that both ski and sno oncoproteins are antagonistic to TGF- β signaling (Luo *et al.*, 1999; Stroschein *et al.*, 1999). Overexpression of ski/sno proteins induces myogenesis and can mediate skeletal muscle fiber hypertrophy (Colmenares and Stavnezer, 1989; Berk *et al.*, 1997). This indicates that expression of ski and sno proteins may antagonize the negative effects of TGF-β signaling on myogenic determination and the regeneration program.

1.4 REGULATION OF TERMINAL DIFFERENTIATION

1.4.1 Cell Cycle and Myogenesis

Decreases in growth factor concentration represents a cue for myoblasts to exit the cell cycle and undergo terminal differentiation. As myoblasts exit the cell cycle, expression of cyclin/cdk (cyclin-dependent kinase) inhibitors and retinoblastoma protein (pRb) are upregulated (Relaix and Buckingham, 1999; Andres and Walsh, 1996; Walsh and Perlman, 1997; Lipinski and Jacks, 1999). The importance of cyclin/cdk inhibitors and pRb has been demonstrated by the fact that overexpression of E1A, which renders pRb inactive, inhibits myogenesis and can permit terminally differentiated myotube nuclei to reenter the DNA synthesis phase (S-phase) of the cell cycle (Tiainen *et al.*, 1996; Mal *et al.*, 2000). Similarly, myoblasts lacking a functional copy of *Rb* re-initiate DNA synthesis upon growth factor stimulation. However, unlike E1A mediated inactivation of pRb, *Rb* null cells are capable of differentiating in the absence of pRb suggesting that p130 and p107 can compensate during differentiation but, are unable to

maintain the differentiated phenotype (Schneider *et al.*, 1994; Novitch *et al.*, 1996). Moreover, both MRF and MEF2 proteins are dependent on pRb expression for full gene activation (Gu *et al.*, 1993; Novitch *et al.*, 1999).

Overexpression of cyclin D1, which is important for the G1-S transition, and increases in cyclin/cdk kinase activity inhibit myogenesis, possibly due to direct binding and inhibition of DNA-binding or, phosphorylation and destabilization of MyoD (Rao *et al.*, 1994; Skapek *et al.*, 1995; Mennerich *et al.*, 1998). The putative cdk1/2 phosphorylation residue is serine-200 which, when mutated to alanine, leads to an increase in MyoD stability and activity (Song *et al.*, 1998). Furthermore, MyoD and Myf5 protein level oscillations during the cell cycle correlate with changes in cyclin expression and cyclin/cdk1/2 activity (Kitzmann *et al.*, 1998). Physiologically, cyclin D1 levels increase upon stimulation of myoblasts with tumor necrosis factor alpha (TNF-α), leading to inhibition of terminal differentiation (Guttridge *et al.*, 1999).

During terminal differentiation, upregulation of cdk inhibitors is important for cell cycle withdrawal (Halevy *et al.*, 1995), resistance to apoptosis (Wang and Walsh, 1996), MyoD stability (Reynaud *et al.*, 1999) and for the induction of *myogenin*, which is necessary for the differentiation program to proceed (Zhang *et al.*, 1999a). Recent data demonstrates a direct link between MyoD and cell cycle regulation (Zhang *et al.*, 1999b; Zhang *et al.*, 1999c). In proliferating myoblasts, nuclear cdk4 binds MyoD and inhibits MyoD-mediated gene expression (Zhang *et al.*, 1999b). Conversely, a short carboxyl-terminal sequence of MyoD can inhibit cyclin/cdk4-dependent phosphorylation of pRb, promoting terminal differentiation (Zhang *et al.*, 1999c). Furthermore, upregulation of the cyclin/cdk inhibitor $p57^{KIP2}$ stabilizes MyoD by blocking cyclinE-cdk2 activity

(Reynaud *et al.*, 1999) and by direct interaction with MyoD (Reynaud *et al.*, 2000). It is clear that a precise balance exists between cell cycle regulation and terminal differentiation.

1.4.2 The Mef2 Family of Transcription Factors

Along with the MRFs, it has been suggested that the myocyte enhancer factor 2 (MEF2) family of transcription factors play a role in myogenesis (for review see Black and Olson, 1998; Naya and Olson, 1999). MEF2 proteins are members of the MADS (MCM1, agamous, deficiens, serum response factor) box-containing family of transcription factors. The MEF2 family consists of four members, MEF2A-D, and they demonstrate a widely distributed pattern of expression. Although much of the information regarding these factors demonstrates their importance in cardiac muscle, they have been shown to form autoregulatory loops with the MRFs and are important for the expression of many muscle-specific genes. Structurally, MEF2 proteins are composed of amino terminal MEF and MADS domains which are responsible for dimerization and DNA binding. The carboxyl terminal domains are important for gene activation and kinase responsiveness (Black and Olson, 1998). Homo- and heterodimers bind an A/T rich DNA sequence element ($C/TTA(A/T)_4TAG/A$) which is found in the promoters of many muscle-specific genes (Black and Olson, 1998).

Several lines of evidence suggest that MEF2 and MRFs synergistically activate gene expression. It is important to note that MEF2 expression is initiated after the onset of differentiation suggesting these factors are involved during later stages of terminal differentiation (Naya and Olson, 1999). At the level of gene expression, full activation of both *MRF4* and *myogenin* promoters require both MRF and MEF2 proteins (Edmondson

et al., 1994; Naidu *et al.*, 1995). *In vitro*, MRF and MEF2 proteins are capable of interacting to activate gene expression by both indirect and direct mechanisms (Naidu *et al.*, 1995; Molkentin *et al.*, 1995). In flies, ablation of the single *MEF2* gene results in an inability of muscle cells to differentiate (Lilly *et al.*, 1995). By contrast, targeted inactivation of the *MEF2C* gene in mice is embryonic lethal due to severe defects in cardiac morphogenesis (Lin *et al.*, 1997). However, no defects in skeletal muscle were noted, possibly due to functional redundancy of the factors. Transgenic mice carrying a *lacZ* reporter gene regulated by MEF2 factors show that MEF2 activity is high during embryonic development but is not detected after birth until the induction of cardiac stress (Naya *et al.*, 1999). Downregulation of MEF2 activity suggests that MEF factors are regulated at a posttranslational level that is currently unknown (Naya *et al.*, 1999).

1.4.3 Mitogen-Activated Signal Transduction Pathways

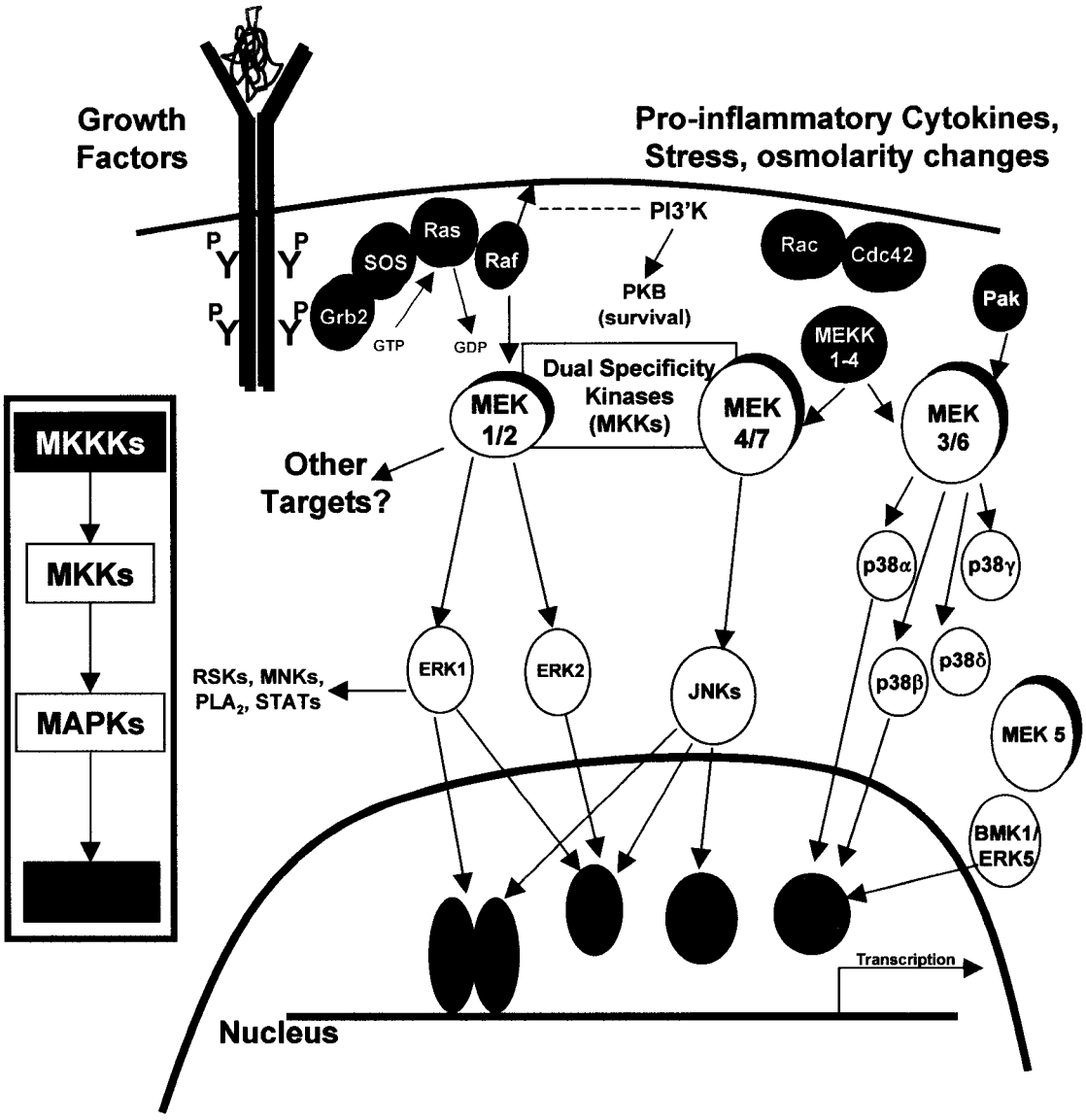
Extracellular stimuli elicit cellular responses via a series of signal transduction cascades. In particular, the mitogen-activated protein kinase (MAPK) pathways are activated by diverse signals such as peptide growth factors, cytokines and extracellular stresses such as ultraviolet (UV) irradiation and changes in osmolarity. Activated MAPK pathways regulate proliferation, differentiation, apoptosis, and morphological changes (for extensive reviews of MAPK signaling see Lewis *et al.*, 1998; Chen *et al.*, 2001). In mammals, three distinct MAPK pathways have been identified. The extracellularly-regulated kinase (ERK; also known as mitogen-activated protein (MAP) kinases) pathway is generally responsive to peptide growth factors and is usually activated in response to proliferative signals. The jun-amino-terminal kinase (JNKs; also known as stress-activated protein kinases (SAPKs)) pathways are activated by cellular stress and

cytokines. In general, JNK pathway leads to activation of the AP-1 (activator protein-1) transcription factor complexes and, in some cell types, is associated with pro-apoptotic signals (Weston and Davis, 2002; Dunn *et al.*, 2002). The p38 pathway (also known as HOG-1 from the yeast hyperosmolarity response pathway) is activated by many of the same stimuli as the JNK pathway and is thought to play roles in G2-M checkpoint control, immune responses and tissue differentiation (Bulavin *et al.*, 2002). The importance of these pathways in regulating cell proliferation is strengthened by the fact that mutants of many individual components leading to constitutive activation lead to oncogenic transformation (Hunter, 1997).

Each MAPK pathway is represented as a module involving several intermediate kinase members. Upstream activators of MAPKs are condsidered MAPK kinases (MAPKK, MKK or also known as MEKs (MAP/ERK kinases)) and MKK upstream activators being MAPK kinase kinases (MAPKKKs, MKKKs or MEK kinases (MEKKs)) (Figure 1.3). Extracellular stimuli initiate MAPK signaling by first activating the small GTPase family of protein kinases. This family includes several members, such as ras, and are constitutively localized to assorted cellular membranes (for review see Matozaki *et al.*, 2000; Ehrhardt *et al.*, 2002). In their inactivated state, these small GTPases are bound with guanosine diphosphate (GDP). In the example of ras, exchange of GDP for guanosine triphosphate (GTP) leads to ras activation and recruitment of downstream kinase targets to the plasma membrane (Matozaki *et al.*, 2000; Chong *et al.*, 2003). Exchange of GDP for GTP is facilitated by interaction with guanine nucleotide exchange factors (GEFs), for example Sos (son of sevenless) (Ehrhardt *et al.*, 2002). By

Figure 1.3: Vertebrate MAPK Signaling Pathways.

Shown is a schematic of the known vertebrate MAPK pathways. On the left of the figure is the typical MAPK module. The colour coding delineates which molecules are considered to be at a certain level of the pathway. In the example shown, growth factor binding results in receptor dimerization, tyrosine kinase domain activation and phosphorylation of several tyrosine residues in the cytoplasmic tail portion of the receptor. These phosphotyrosine residues recruit SH2 domain molecules, such as Grb2, to the membrane. Recruitment of a Grb2/SOS complex in close proximity to ras leads to exchange of GDP for GTP thus, activating ras. Activatied ras recruits raf to the membrane and ras-mediated phosphorylation leads to raf activation. Activated raf activates MEK1/2 by phosphorylation on two serine residues. MEK1/2 are considered dual-specificity kinases and activate the ERKs by phosphorylation of both a tyrosine and threonine residue. Upon activation, ERKs translocate to the nucleus and target several substrates such as components of the AP-1 transcriptional complex and members of the ternary complex factors (TCFs), such as ELK-1. Phosphorylation of transcription factors by the MAPKs can lead to increased transcriptional activity, thereby permitting a cellular responses to specific extracellular signals. The potential nuclear targets of the JNK, ERK5 and p38 pathways are also shown. While several nuclear substrates are targets of multiple pathways, there are pathway-specific molecules that when targeted, permit highly specific cellular responses. Although not shown, there are several distinct MAPK family members, many of which are alternatively spliced yielding multiple isoforms at all levels of the pathways. These multiple family members provide a wide variety of tissue-specific responses under conditions of similar extracellular stimulation.



Signaling through these pathways regulate cell proliferation, differentiation, cell survival and apoptosis

contrast, stimulation of ras GTPase activity, thereby inactivating ras, is mediated by interaction with GTPase activating proteins (GAPs) (Bernards, 2003).

In the case of peptide growth factors, ligand binding induces receptor dimerization. Dimerization of receptor tyrosine kinases (RTKs) leads to cross-phosphyorylation and activation of a cytoplasmic tyrosine kinase domain, resulting in receptor activation (for extensive review of RTK activation see Schlessinger, 2000). Activated receptors autophsophorylate tyrosine residues located in their cytoplasmic tail domains. Phosphotyrosine (pTyr) residues serve as docking sites for molecules containing domains recognizing this modified residue (Sudol, 1998; Pawson *et al.*, 2001; Vidal *et al.*, 2001). One important example is the recruitment of a Grb2/Sos complex to an activated RTK. Grb2 represents an example of an adaptor protein and binds pTyr residues via its src homology-2 (SH2) domain. Interaction of Grb2 with Sos occurs via a src homology-3 (SH3) domain. Therefore, recruitment of Grb2 to pTyr residues on activated RTKs brings Sos, a ras-GEF, into close proximity to ras. Sos induces the GDP-GTP exchange reaction, thereby activating ras kinase activity (Ehrhardt *et al.*, 2002).

Recruitment to the plasma membrane and serine phosphorylation of raf by activated ras leads to raf activation. Raf represents a MEK kinase (MEKK or MAPK kinase kinase (MKKK)) and once activated, raf activates MEK1/2 (MAP/ERK kinase 1 and 2, MKK1/2 or MAPKK1/2) by phosphorylating two serine residues in the C-terminal activation loop domain of MEK1/2 (reviewed in Chong *et al.*, 2003). MEKs are considered dual specificity kinases due to their ability to phosphorylate substrates on both threonine (T) and tyrosine (Y) residues. Upon activation, MEKs phoshorylate and activate the MAPKs. Activation of the MAPKs leads to the phosphorylation of several

membrane-bound, cytoplasmic and nuclear substrates (Lewis *et al.*, 1998). Nuclear translocation of activated MAPKs is important for altering gene expression as serine/threonine phophorylation of transcription factors affects transcriptional activity and, therefore, patterns of gene expression (Chen *et al.*, 2001).

While there are numerous shared targets for the MAPKs, it is clear each pathway targets specific subsets of factors (Lewis *et al.*, 1998). The general requirement for MAPK phosphorylation is the presence of a proline (P) residue at the +1 position from the serine or threonine residue that gets phosphorylated (Clark-Lewis *et al.*, 1991; Alvarez *et al.*, 1991). However, the presence of an additional proline residue at –2 results in increased targeting preferences for all MAPKs (Clark-Lewis *et al.*, 1991; Alvarez *et al.*, 1991). Specificity of activation is achieved by regulation of substrate recognition and binding. Each MAPK has a recognition motif that permits docking with and phosphorylation of downstream targets (Chen *et al.*, 2001). The importance of substrate recognition has been observed by domain swapping experiments in which different domains of the p38 and ERK1 (p44) were interchanged and both upstream activation and downstream substrate targeting were analyzed (Brunet and Pouysségur, 1996). Substitution of the N-terminal domains affected the nature of upstream activation whereas the C-terminal domains regulated substrate recognition and phosphorylation. Importantly, one chimeric molecule permitted the redirection of stress signals to mitogenic outputs (Garrington and Johnson, 1999). These results demonstrated that while the MAPKs have distinct –TXY- MEK phosphorylation motifs (where X is E (glutamic acid) in ERKs, G (glycine) in p38 isoforms, and P (proline) in JNKs), docking of upstream activators determines specificity of activation. Similarly, MAPK targets are

determined by binding domains with phosphorylation of S/T residues occurring promiscuously (Garrington and Johnson, 1999).

An additional level of complexity with regard to MAPK signaling involves a series of molecules that serve as scaffolding components (Burack and Shaw, 2000). These scaffold proteins permit direct linking of different MAPK pathway intermediates. Examples of scaffolding proteins are MP-1 (Schaeffer *et al.*, 1998), JIP1 (Whitmarsh *et al.*, 1998) and a family of molecules called the 14-3-3 proteins (Tzivion *et al.*, 2001). Scaffold molecules aggregate specific upstream and downstream kinases serving to regulate the precise pathways activated in response to extracellular signals. As observed with domain swapping experiments altering cellular outputs, recent experiments have clearly demonstrated the importance of scaffold interactions in a similar manner. Swapping scaffold interaction domains resulted in the ability of upstream stimuli to inappropriately activate downstream targets of other pathways (Park *et al.*, 2003). The ability to alter substrate targeting clearly demonstrates an intricate network of how cells respond to diverse extracellular signals. Furthermore, changes in expression patterns of the multitude of signaling components can permit cell type-specific responses to identical extracellular stimuli.

1.4.4 Growth Factors and Signal Transduction in Skeletal Muscle

The determination, maintenance and activation of the myogenic program during development is regulated by factors such as Shh, BMPs, FGFs and Wnts. To gain an understanding of how extracellular signals regulate myogenesis, several studies have been carried out using myoblast cell lines *in vitro*. Treatment of cells with growth factors and cytokines leads to the activation of several intracellular kinase pathways which

ultimately lead to changes in gene expression, cell survival and cellular morphology (for review see Hunter, 2000). Many distinct mechanisms have been elucidated to explain how growth factors are able to repress or stimulate the myogenic program.

Protein kinase C (PKC) activity is increased in response to mitogenic stimulation. Overexpression of activated PKC represses MRF-mediated transcription of muscle-specific reporter vectors and terminal differentiation. Transcriptional activation and DNA-binding are regulated by the direct phosphorylation of a threonine residue in the basic domain of myogenin (Li *et al.*, 1992a). Although this threonine residue is conserved in all four MRFs, PKC phosphorylation is specific for myogenin suggesting that PKC-mediated regulation of myogenesis involves other pathways (Hardy *et al.*, 1993).

Binding of ligands to cell-surface receptors initiates a cascade of events which leads to the activation of ras^{p21}. Overexpression activated ras^{p21}in 10T1/2 mouse fibroblasts inhibits MRF-mediated differentiation without altering DNA-binding or the inherent transcriptional activation properties of the MRFs (Kong *et al.*, 1995). Interestingly, inhibition of MEK and rac/rho kinase pathways, which are activated by ras, do not rescue myogenesis suggesting these pathways are not involved in regulating terminal differentiation (Ramrocki *et al.*, 1997), although overexpression of activated and dominant inhibitory forms of rac inhibit or stimulate myogenesis, respectively (Heller *et al.*, 2001). Experiments examining the MAPK pathway demonstrate that inhibiting MEK signaling alleviates the repressive effects of FGF on myoblast differentiation (Weyman and Wolfman, 1998), whereas expression of activated c-raf inhibits myogenesis (Winter and Arnold, 2000). Furthermore, overexpression of the MAPK phosphatase, MKP-1,

which is normally upregulated during differentiation, is important for inhibiting MAPK activity and permitting differentiation (Bennett and Tonks, 1997). It should be noted that later stages of differentiation require MKP-1 downregulation for myoblast fusion and myotube formation (Bennett and Tonks, 1997). Taken together, it is clear that increases in MAPK signaling are required for transmitting growth signals and decreases in MAPK activity is required for myogenesis to proceed.

Insulin-like growth factors (IGFs) are known to positively regulate myogenesis. IGF stimulation leads to an increase in phosphatidylinositol 3'-kinase (PI3'K) activity. Dominant negative forms of PI3'K or, inhibition of PI3'K activity using synthetic inhibitors, are able to block IGF-mediated differentiation (Kaliman *et al.*, 1996; Coolican *et al.*, 1997; Jiang *et al.*, 1998). When IGF signaling is blocked, cells maintain high levels of Id proteins and are unable to upregulate $p21^{Cip1}$ for cell cycle withdrawal (Kaliman *et al.*, 1996). Conversely, expression of activated PI3'K is able to induce differentiation suggesting a direct role for PI3'K in myogenesis (Jiang *et al.*, 1998).

The lipid products resulting from stimulation of PI3'K activity serve to activate protein kinase B (PKB/Akt). During differentiation, PKB expression is upregulated and its activity is important for myocyte survival (Fufio *et al.*, 1999). Expression of a dominant negative form of PKB inhibits PI3'K and IGF stimulation of myogenesis indicating PKB lies downstream of these signals (Jiang *et al.*, 1999). Surprisingly, activated PKB is able to phosphorylate Raf, rendering the Raf/MEK/MAPK pathway inactive (Zimmermann and Moelling, 1999). Although this inhibition is important during differentiation, overexpression of activated PKB is unable to force differentiation under

growth conditions suggesting the involvement of mediators that are specifically expressed at the onset of myogenic differentiation (Rommel *et al.*, 1999).

In many cell lines, the absence of extracellular growth factor stimulation leads to apoptosis indicating that pathways exist that are essential for cell survival. Although platelet-derived growth factor (PDGF) and IGF elicit opposite responses in myoblast cell lines, either factor on its own is sufficient to prevent apoptosis (Lawlor *et al.*, 2000). Two distinct pathways are utilized indicating that cell survival can be mediated by separate mechanisms (Lawlor *et al.*, 2000). What is surprising is that myoblasts stimulated with PDGF, which is mitogenic, produce a transient PKB activation and prolonged ERK activation. By contrast, IGF leads to transient ERK activation and prolonged PKB activity suggesting that the decision to proliferate is dependent upon the length of time that the MAPK pathway is active (Lawlor *et al.*, 2000). This mechanism has been proposed for regulating proliferation versus differentiation decisions in the pheochromocytoma cell line, PC12, although prolonged ERK activity leads to differentiation (Marshall, 1995). It remains to be seen what molecular events occur in myoblasts to elicit these distinct responses to extracellular cues.

MEF2 proteins are positively regulated by both p38 stress-activated and MKK5/BMK1 kinase pathways (Han *et al.*, 1997; Kato *et al.*, 1997; Yang *et al.*, 1999; Zhao *et al.*, 2000). The finding that MEF2 factors represent downstream targets of these pathways suggests that activation of MEF2 transcriptional activity is an important step during myogenesis. Indeed, overexpression of p38 isoforms or, upstream activators, stimulates myogenesis (Lechner *et al.*, 1996; Zetser *et al.*, 1999). It is interesting to note that the gamma isoform of p38 (SAPK3-beta/ERK6) is highly expressed in skeletal

muscle. Although expression of this kinase is upregulated upon differentiation, it does not appear to phosphorylate MEF2 proteins and therefore, its function remains unclear (Lechner *et al.*, 1996; Wang *et al.*, 1997a).

Slow and fast muscle fibers differ in their metabolic properties and the panel of contractile proteins that they express. Since intracellular levels of calcium are regulated by contraction speeds, it has been hypothesized that calcium activated signal transduction pathways are important for fiber-type specification (Naya and Olson, 1999). Calcineurin, which is a calcium-activated protein phosphatase, activates the NFAT (nuclear factor of activated T-cells) transcription factors by dephosphorylation. This permits nuclear translocation of NFATs where they interact with other transcription factors and activate gene expression (Crabtree, 1999). Interestingly, treatment of animals with cyclosporin A, an inhibitor of calcineurin, or overexpression of calcineurin in muscle causes a shift from fast to slow fibers (Chin *et al.*, 1998; Dunn *et al.*, 1999; Naya *et al.*, 2000). One potential mechanism by which NFATs are thought to alter fiber-type specific gene expression is by interaction with MEF2. Response of T-cells to changes in intracellular calcium levels is mediated by MEF2 proteins (Youn *et al.*, 1999; Mao *et al.*, 1999; Blaiser *et al.*, 2000) and many fiber-type specific gene promoters contain both MEF2 and NFAT binding sites (Naya and Olson, 1999).

1.4.5 Functional Protein-Protein Interactions

Growth factor stimulation increases AP-1 (fos/jun)-dependent gene expression. Expression of the *c-fos* gene is mediated by binding of the serum-response-factor (SRF) to a serum-response-element (SRE) in the *c-fos* promoter. During differentiation, *c-fos* gene is downregulated leading to decreases in AP-1-meditated gene activation. MRF-

mediated repression of c-fos expression requires an E-box element that overlaps the SRE in the *c-fos* promoter (Trouche *et al.*, 1993). It is unclear whether MRF-mediated repression represents a competition for binding sites or, if direct interaction between MRFs and SRF is required (Groisman *et al.*, 1996). Moreover, the finding that c-jun can interact directly with MyoD and inhibit MRF-mediated gene expression suggests that AP-1 and MRFs form an autoregulatory loop to control myogenesis (Bengal *et al.*, 1992; Li *et al.*, 1992b).

MRFs require dimerization with E-proteins in order to bind DNA and activate gene expression. One potential level of regulation involves the Id factors. Id molecules contain a helix-loop-helix motif but lack a basic DNA-binding domain (Langlands *et al.*, 1997 and references therein). Id levels increase upon stimulation of cells with growth factors and dimerization of Id proteins with MRFs or E-proteins prevents DNA binding and MRF-mediated gene expression. Expression of a MyoD-E47 fusion protein is resistant to Id regulation demonstrating the functional significance of Id proteins in regulating MRF-mediated gene expression and terminal differentiation (Neuhold and Wold, 1993). Alterations in E-protein availability has also been shown to occur by the MyoR bHLH factor (Lu *et al.*, 1999). MyoR is expressed specifically in skeletal muscle and its expression is downregulated upon differentiation. Unlike Id/E-protein dimers, MyoR/E-protein dimers bind DNA and serve to repress gene expression (Lu *et al.*, 1999).

Although the Mos protooncogene is generally regarded as an upstream activator of the MAPK signal transduction pathway, activation of the Mos protooncogene in muscle cells stimulates myogenesis (Leibovitch *et al.*, 1995). Mos-mediated myogenic stimulation occurs at two levels. First, activated Mos stimulates dimerization of MyoD

and E12 and second, MyoD directly interacts with Mos, inhibiting downstream Mos-mediated activation of the MEK/MAPK pathway (Lenormand *et al.*, 1997; Solhonne *et al.*, 1999). These results suggest that alterations in the dimerization status of the MRFs are important levels of myogenic regulation. Indeed, interaction of MRF/E-protein dimers with muscle LIM protein dramatically increases MRF/E-protein gene activation and stimulates myogenic differentiation (Kong *et al.*, 1997).

1.4.6 Chromatin-Remodeling and MRF Function

Several molecules have been shown to interact with MyoD. Of particular interest is the regulation of MyoD activity by p300/CBP and PCAF. These molecules are vital for gene activation by altering the acetylation status of histone cores in DNA (for reviews see Berger, 2002; Schreiber and Bernstein, 2002). The transactivation domain of MyoD and the MADS domain of MEF2 proteins interact with p300, which initiates cell cycle arrest and differentiation (Puri *et al.*, 1997a; Sartorelli *et al.*, 1997). Interestingly, the histone acetyltransferase (HAT) activity of p300 is dispensable for MRF-mediated gene expression and only serves to attract PCAF to the promoters of muscle-specific genes (Puri *et al.*, 1997b). Significantly, MyoD transcriptional activation requires the acetylation of several lysine residues located just amino terminal of the basic DNA-binding domain by PCAF (Sartorelli *et al.*, 1999). In light of the fact that under growth conditions MyoD interacts with N-CoR (Bailey *et al.*, 1999), this suggests a molecular switch during activation of the myogenic program. Under proliferating conditions, MyoD association with N-CoR serves to attract histone deacetylases (Bailey *et al.*, 1999). As differentiation proceeds, N-CoR levels decrease and p300/PCAF complexes initiate MyoD-meditated gene expression. Indeed, the fact that MyoD has two domains

necessary for chromatin remodeling lends support to this type of regulation (Gerber *et al.*, 1997).

1.5 THESIS OBJECTIVES

It is clear that a great deal of information has been obtained regarding many aspects of skeletal muscle development. The myogenic regulatory factors represent an ideal paradigm for the study of cell lineages and the molecular events required for the establishment of a terminally differentiated tissue. However, several questions remain concerning aspects of determination, proliferation and terminal differentiation. In particular, the molecules responsible for the *de novo* activation of *MyoD* and *Myf5* are unknown. Similarly, regulation of MRF activity during proliferation and terminal differentiation are poorly understood due to a lack of myoblast specific genes that have been identified to date. Although several signal transduction pathways and protein-protein interactions regulating MRF expression and activity have been described, how these processes are integrated represents a major challenge in muscle research. Moreover, coordination of cell cycle and terminal differentiation is complex and we are only now beginning to understand the multitude of factors involved.

The objective of this research was to determine: i) the nature of myogenic lineage determination and ii) the underlying molecular mechanism of growth factor-mediated repression of MRF transcriptional activity. To examine the nature of myogenic lineage determination and maintenance, fibroblast cell lines lacking functional copies of *MyoD* and *Myf5* genes were generated and transfection studies were performed. The data demonstrates that *MyoD* and *Myf5* are dispensable for terminal differentiation but at least one of these factors is required for lineage maintenance. These fibroblast cell lines have

been used by collaborators for studies examining the details of MyoD-mediated activation of the myogenic program (Bergstrom *et al.*, 2002). The data contained within Chapter 3, along with some new experiments, will be submitted for publication (Perry, R.L.S., Laing, M.A., Krastel, K. and Rudnicki, M.A.) (Abstract presented at Myogenesis conference, Banff, Alberta, Canada, May 30-June 4, 2003. Abstract #35).

Experiments detailed in Chapters 4 and 5 clearly show that the MAPK pathway, when activated, negatively regulates myogenesis. The data shows that the MAPK signaling intermediate MEK1 represses MyoD-mediated gene expression by binding to a transcriptional complex containing MyoD and its heterodimerization partner HEB. This interaction likely requires a cofactor and represents a mechanism by which myogenesis is inhibited without a concomitant loss of lineage identity. Experiments also revealed a novel mechanism of regulation via the muscle-specific and cell cycle-dependent loss of HEB. Data in Chapters 4 and 5 has already been published (Perry *et al.*, 2001) and experiments focused on the importance of HEB are currently underway (Parker, M. and Rudnicki, M.A.) and will be submitted for publication (Parker, M.H., Perry, R.L.S. and Rudnicki, M.A.) (Abstract presented at Myogenesis conference, Banff, Alberta, Canada, May 30-June 4, 2003. Abstract #106).

The work presented here provides increased insight into the myogenic lineage, has uncovered a novel mechanism of MAPK-mediated regulation of terminal differentiation and discovered a novel and potentially critical mechanism of myogenic regulation by the cell cycle-dependent loss of the MRF dimerization partner HEB.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture and Transfections

Cell Culture. C3H10T1/2 (10T1/2) mouse fibroblasts and C2C12 mouse myoblast cell lines were obtained from the American Tissue Culture Collection (ATCC; CCL-226 and CRL-1772). Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; InVitrogen) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin and fungizone (Growth Medium (GM)), and maintained at 37^{O}C with a humidified 5% CO_2 atmosphere. For induction of myoblast terminal differentiation, growth medium was removed, cells were washed with phosphate buffered saline (PBS) and refed with DMEM containing 2% horse serum (HS) supplemented with penicillin/streptomycin and fungizone (differentiation medium (DM)).

Transfections. Transfections were performed using the calcium phosphate method. Briefly, one day prior to transfection, cells were seeded at $1x10^5$ or $2x10^5$ on 10-cm tissue culture dishes (Corning). The following day, cells were refed with 10 mls growth medium three hours prior to the start of transfection. Plasmids were diluted to the appropriate concentrations in 450 µls of ddH_2O and 50 µls of $CaCl_2$ was added. This mixture was added dropwise to tubes containing 500 µls of 2X HeBS [pH 7.15] (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4) while slowly shaking on a vortexer. After 20-30 minutes incubation at room temperature, transfection solutions were added dropwise to the culture medium and cells were returned to the incubator for approximately 16 hours. Transfection medium was aspirated, cells were washed twice with PBS and refed with growth medium. Twenty-four or forty-eight hours later, cells were washed with

PBS and refed with differentiation medium (DM). Forty-eight hours after transfer to DM, cells were harvested or chemically fixed. Generally, efficiences ranged from 30-50% as determined by green fluorescent protein (GFP) expression with variability between transfection sets rather than within a set of transfection plates.

Generation of Double Knock-Out Mouse Fibroblasts. Timed matings were set-up and embryos were harvested at embryonic day 12.5. Embryos were dispersed by mechanical disruption followed by trypsinization and tituration with organs and heads being removed. Individual embryos were plated onto 100-mm tissue culture plates with growth medium. Genotyping was done by Southern blotting as previously described (Rudnicki *et al.*, 1992; Braun *et al.*, 1992). Cultures were expanded once and freezer stocks generated. Double knock-out (DKO) mouse embryonic fibroblasts (MEFs) that were *MyoD-/-:Myf5-/-* were thawed and immortalized essentially using the C3H10T1/2 protcol (Reznikoff *et al.*, 1973). Briefly, cells were seeded at $1x10^6$ per 100-mm plate, refed with growth medium after 5 days and trypsinized and seeded at $1x10^6$ on the tenth day. This regiment was maintained until cells reached crisis (approximately passage 13). For the next two passages cells were refed every 5 days and passaged when confluent (generally 12-14 days after seeding). Cells were then seeded at $1x10^5$ or $2x10^5$ and passaged when 75-85% confluent. Clonal density plates were seeded (500 cells/100-mm plate) and colonies were isolated, expanded and subcloned. Specific clones were selected on the basis of transfection efficiency, as ascertained using a β-galactosidase staining method, and MyoD-mediated conversion to the myogenic lineage, as assessed by MF20 immunocytochemistry. It should be noted that the ability of cells to convert to the

myogenic lineage was solely based on transfection efficiency and did not appear to occur in subsets of cells. Two clones were used for further study: 2C5/7 and 4C5/2.

Generation of MyoD-ER Clones

DKO clones that were shown to convert to the myogenic lineage were transfected with the MyoD-ER plasmid (Hollenberg *et al.*, 1993) that had the PGK-puromycin resistence cassette cloned into the vector. Cells were selected with 4 μg/ml puromycin and 48 colonies were selected. Each colony was exposed to 10^{-7} M β-estradiol and assessed for their ability to terminally differentiate as measured by MF20 immunocytochemsitry. Several clones were chosen for further study: 2C5/7A-A/C5 and 4C5/2B-B/A4. It should be noted that several clones were examined and all demonstrated an ability to convert to the myogenic lineage and terminally differentiate.

C2C12 Synchronization Procedure. To synchronize C2C12 myoblast cell cultures established protocols were followed (Kitzmann *et al.*, 1998). Briefly, cells were seeded at $5x10^4$ per 60-mm dish or $2x10^5$ per 100-mm plate 24-36 hours before the start of synchronization. Proliferating cells were washed with PBS and refed with DMEM lacking cysteine and methionine supplemented with 1% dialyzed FCS, L-glutamine, penicillin/streptomycin and fungizone. Cells were returned to the culture incubator for 36 hours. Medium was removed and the cells were refed with complete growth medium and returned to the incubator for 1 hour. Hydroxyurea (HU) was added to the culture medium to a final concentration of 1 mM and the cells were incubated for an additional 15 hours. Medium was aspirated, cells were washed with 3 changes of PBS and refed with complete growth medium. This step represents the G1-S boundary and was considered time 0 for synchronization studies. Cells were harvested at specific time

points after HU release and extracted for protein or fixed with 90% methanol and processed for immunocytochemistry. In some cases, cells were released at time 0 and refed with differentiation medium with or without the MEK1 inhibitor U0126 (Promega).

2.2 Plasmids, Cloning Procedures and GST-Protein Isolation

Plasmids. Plasmids encoding the MRFs have been described elsewhere (Davis *et al.*, 1987; Braun *et al.*, 1989a; Braun *et al.*, 1990; Rhodes and Konieczny, 1989; Wright *et al.*, 1989). MyoD deletion mutants were a kind gift of Dr. Stephen Tapscott and have been described elsewhere (Gerber *et al.*, 1997). MRF expression plasmids utilize the Moloney Sarcoma Virus long-terminal-repeat (MSV-LTR) which is constitutively active in the cell lines used for these studies. For control transfections, vectors lacking cDNA inserts were used to ensure the results were controlled for promoter squelching. Plasmids encoding the wild-type, dominant negative (K97M) and activated (ΔN3 S218D/S222E) versions of human MEK1 were a kind gift from Dr. Natalie Ahn and have been described elsewhere (Mansour *et al.*, 1994). Expression plasmids containing carboxyl terminal truncated forms of MyoD were obtained by PCR using full-length or Δ63-99 MyoD plasmids as templates and inserts were coned into the pCDNA3 (InVitrogen) expression vector. MyoD/VP16 fusion vectors were produced by overlapping PCR using the Gal4-VP16 fusion expression plamid (Sadowski *et al.*, 1988) and full-length MyoD as templates. The reporter vectors used for these studies are the 4RtkCAT, which contains four myogenic E-box motifs from the right hand MLC enhancer element and has been described elsewhere (Weintraub *et al.*, 1991). The MLC1 promoter/enhancer CAT reporter was a kind gift from Dr. Nadia Rosenthal and has been described elsewhere (Grieshammer *et al.*, 1992). GST fusion vectors used for this study (Figure 2.1 A) were

either produced in the laboratory by PCR amplification and cloned into the pGEX4T-1 (Pharmacia) or were kind gifts of Drs. John Hassell and Atsushi Asakura. GST-MyoD and GST-E12 represent the full-length sequences from mouse and human cDNAs, respectively. GST-jun represents amino acids 5-89 and GST-Elk-1 represents amino acids 307-428 (Figure 2.1A). MEF2C and 2X MEF-CAT were kind gifts of Dr. John McDermott and Pea3 and 4X Pea3-CAT were kind gifts of Dr. John Hassell.

GST Protein Isolation. Isolation of GST-fusion proteins was done as described elsewhere (Smith and Johnson, 1988) using the BL21(DE3) strain of *E. coli*. Briefly, bacterial cultures were initiated in LB broth containing 50 μg/ml ampicillin and allowed to grow for 5-6 hours. Cultures were stimulated with a final of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1.5 to 2.5 hours and bacterial pellets were obtained by centrifugation. Bacteria were washed once with STE (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH8.0]) and subjected to centrifugation. Bacteria were resuspended in 5 mls PBS^+ (PBS containing 1mM PMSF, and 10 μg/ml each of aprotinin, pepstatin A, and leupeptin). Bacterial suspensions were lysed by three rounds of sonication, divided into equal aliquots and Triton X-100 was added to a final concentration of 1% (v/v). Lysates were extracted on ice for 30 minutes with periodic and vigorous vortexing. Lysates were cleared by centrifugation and supernatants transferred to fresh eppendorf tubes. Glutathione-conjugated sepharose beads (Phamacia), presoaked overnight in PBS, were washed 3X with PBS^+ (50 % slurry in PBS^+), added to the lysates and binding reactions were carried out at $4^{O}C$ overnight on a rotating platform. Beads were pelleted by centrifugation, washed several times with PBS^+ and fusion proteins were eluted with several changes of elution buffer (50 mM Tris-HCl, 1 mM EDTA containing 10mM

reduced glutatione, [pH 8.0]). Elutions were carried out until protein was no longer detected by Bradford measurement. Protein concentrations were determined by the modified Bradford method (BioRad) and confirmed by comparing with a BSA standard viewed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue (R-250) staining. Purified GST-fusion proteins used for these anaylses are shown (Figure 2.1 B).

Molecular Biological Techniques. All techniques considered standard molecular biological methods (DNA isolation, restriction enzyme digestions, ligations, PCR amplification, etc.) were done as prescribed by the product manufacturers or as indicated in standard protocol manuals.

2.3 Cell Extraction and Fixation

Cell Extractions. Cells were washed twice with ice-cold PBS-FV (PBS containing 100 mM sodium fluoride and 1 mM sodium vanadate). Cells were scraped in 1 ml of PBS-FV and cell pellets were resuspended in an appropriate volume of NP-40 lysis buffer (0.5% NP-40 (v/v), 50 mM Tris-HCl [pH8.0], 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA, 0.1 M sodium fluoride containing protease inhibitors (1 mM PMSF, and 10 μg/ml pepstatin A, leupeptin , aprotinin and 1 mM sodium vanadate)). Cells were extracted on ice with periodic vortexing for 30-50 minutes and lysates were cleared by centrifugation. Supernatants were transferred to fresh tubes and either processed immediately or stored at $-80^{O}C$.

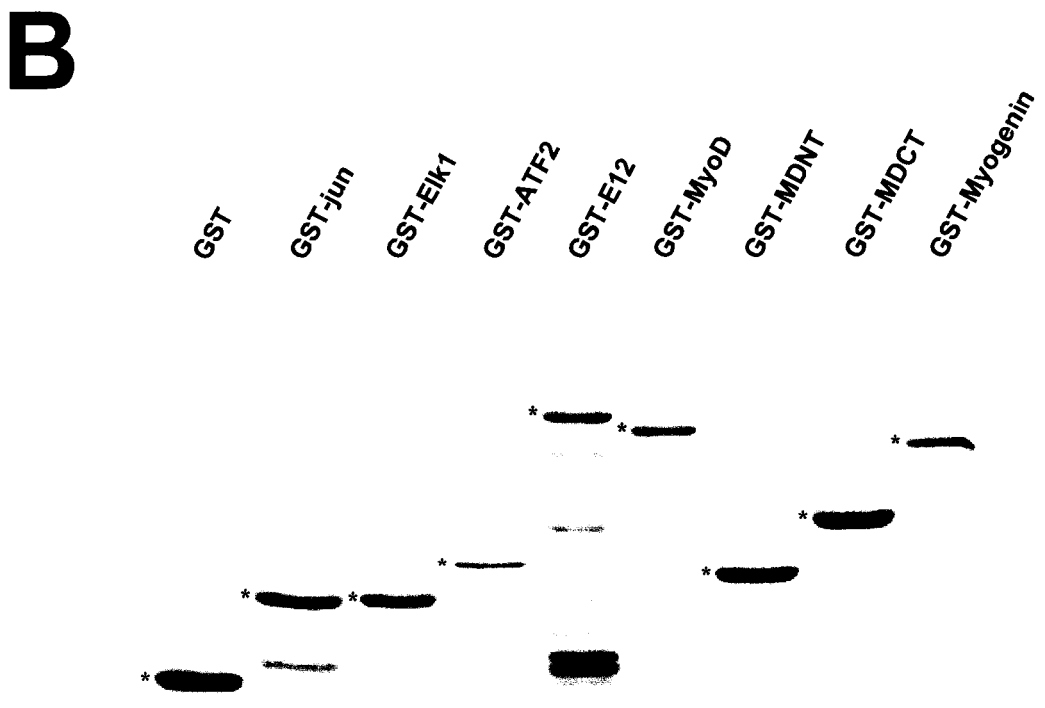
Nuclear versus Cytoplasmic Cell Fractionation. Cells were washed and harvested as described above for whole-cell lysate production. Cell pellets were resuspended in Buffer A (10 mM HEPES [pH 7.8], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM

Figure 2.1: Coomassie stained gel of GST-fusion proteins used for thesis studies.

(A) Schematic representation of GST-fusion proteins used for analyses. The amino acids of each individual fusion protein are indicated on the right. Full-length proteins fused to GST are indicated by FL. Plasmids encoding control GST, GST-ATF2, GST-Elk1 and GST-jun were kindly provided by Dr. John Hassell and GST-E12 was kindly provided by Dr. Atsushi Asakura. See text for details on production of GST-MyoD-FL, GST-MyoD-NT (amino terminal amino acids 1-99) and GST-MyoD-CT (carboxyl terminal amino acids 174-318).

(B) Coomassie stained SDS-PAGE (10%) gel of purified GST-fusion proteins. Purified protein concentrations were determined using the modified Bradford method (BioRad) and 2 μg of protein was loaded onto the gel. The specific fusion of interest is marked with an asterisk with GST representing approximately 27 kDa (Relative Molecular Weight (M_w)).


A
1
220
GST
GST-jun
GST-Elk1
GST-ATF2
GST-E12
GST-MyoD
GST-MDNT
GST-MDCT
GST-Myogenin
a.a 5-89
a.a 307-428
a.a 19-96
a.a 1-648 (FL)
a.a 1-318 (FL)
a.a 1-99
a.a 174-318
a.a 1-224 (FL)


B
GST
GST-jun
GST-Elk1
GST-ATF2
GST-E12
GST-MyoD
GST-MDNT
GST-MDCT
GST-Myogenin

PMSF, 0.1% NP-40, 0.2 mM sodium vanadate with protease inhibitors) and extracted with mild mixing on ice for 30 minutes. Lysates were centrifuged at 4°C and supernatants, representing the cytoplasmic fraction, were transferred to fresh eppendorf tubes. Nuclei were washed with one change of Buffer A and extracted in Buffer C (20 mM HEPES [pH 7.8], 25 % glycerol, 420 mM NaCl, 1.5 mM $mgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.1% NP-40, 0.5 mM sodium vanadate with protease inhibitors) and incubated on ice for 20 minutes with periodic vortexing. Nuclear lysates were cleared by centrifugation and supernatants were transferred to fresh eppendorf tubes and diluted with Buffer D (20 mM HEPES [pH 7.8], 20 % glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.2 mM sodium vanadate and protease inhibitors) to a final concentration of 150 mM NaCl. Samples were stored at –80°C until processing.

RNA Extraction and Northern Blotting

Culture medium was aspirtated and cells were washed with cold PBS-FV. Cells were scraped in 1 ml of PBS-FV, transferred to eppendorf tubes and pelleted. Cell pellets were extracted using the Trizol (InVitrogen) method as per manufacturers instructions with the exception that an additional precipitation step was added at the end of the protocol. RNA was resuspended in DEPC-treated water and stored at –80°C. Quantitation was done by 260/280 spectrophotometry and Northern blots were done as previously described (Sabourin *et al.*, 1999). Northern blots were probed as indicated on each figure. Values were obtained using a Moecular Dynamics Phosphorimager.

2.4 Reporter Assays and Immunologically-Based Methods

Reporter Assays. Cells transfected with the indicated plasmids were washed twice with PBS, scraped in 1 ml of PBS and cell pellets collected by brief centrifugation. PBS supernatants were aspirated, cell pellets were resuspended in CAT Assay buffer (250 mM Tris-HCl [pH 7.5]) and subjected to three rounds of freeze/thaw lysis. Extracts were cleared by centrifugation and lysates were transferred to fresh tubes and stored at $-20^{O}C$ until assayed. CAT assays were carried out as previously described (Gorman *et al.*, 1982). Briefly, assays were performed using an equal volume of extract and incubated at $37^{O}C$ for 1 hour (10 µl of 10mM acetyl coenzymeA (Amersham-Pharmacia), 4 µl of [^{14}C]-chloramphenicol (D-threo-[dichloroacetyl-1,2-^{14}C]; 50-60 mCi/mmol, 0.05 mCi/ml (NEN Life Science Products), in a final reaction volume of 180 µl). Radiolabelled chloramphenicol and acetylated products were extracted with ethyl acetate, centrifuged for 5 minutes and the upper organic phases were transferred to fresh eppendorf tubes and dried using a Savant vacuum centrifuge. Pellets were resuspended in a small volume (25 µl) of ethyl acetate and spotted onto thin-layer chromatography (TLC) plates (Whatman). TLC plates were run in 95:5 chloroform:methanol (v/v) until the solvent front reached the top of the plate (generally 25-30 minutes). TLC plates were dried, wrapped in resinite and quantitated using a Molecular Dynamics Phosphorimager. Percent acetylation calculations were done and CAT activities were normalized to protein concentrations obtained using the Modified Bradford method (BioRad) with BSA for standard curve calculations. Relative CAT activities were determined as compared to reporter alone and data presented in graphical form is plotted as relative CAT activity with the error bars representing the standard error of the mean (±SEM).

Immunoblotting and Antibodies. Extracts prepared for immunoblotting were quantitated using the Bradford method and equal concentrations of proteins were mixed with 2X Laemmli sample buffer containing a final concentration of 0.2 M dithiothreitol (DTT). Samples were boiled for four minutes and equal protein amounts separated using 10 or 12 % SDS-PAGE. Proteins were electrotransferred to Immobilon-P nylon membranes (Millipore). Blots were washed several times with PBS, blocked for one hour at 37°C in 5% nonfat dry milk in PBS (5% NFDM) and incubated for one hour at room temperature (rt) with primary antibodies diluted in 5% NFDM. Blots were washed with three changes of PBS and incubated for 1-2 hours at rt with a 1:1000 dilution of HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (BioRad). Blots were washed four times with PBS over forty minutes and immune complexes were detected using the Enhanced Chemiluminescence (ECL) method (Amersham). Antibodies and dilutions used for these studies were as follows: detection of MyoD by immunoblotting rabbit polyclonal (Santa Cruz; C-20), detection of MyoD by immunoflourescence (clone MoAb 5.8A; PharMingen), mouse monoclonal anti-myogenin (clone F5D; Developmental Studies Hybridoma Bank), anti-HA monoclonal (clone 12CA5; Roche), p38 rabbit polyclonal (N-19; Santa Cruz), ERK rabbit polyclonal (C-16; Santa Cruz), JNK 1 monoclonal (clone G151-333; PharMingen). For immunoblotting, all primary antibodies were used according to manufacturers instruction and anti-myogenin supernatant was used at 1:5 or 1:10 depending upon the preparation.

Immunoprecipitations (IP) and IP-Kinase Assays

For immunoprecipitations, 250-500 μg of cell extract was diluted to 650 μl with NP-40 lysis buffer (supplemented with phosphatase and protease inhibitors) and 0.5-1.0

μg of antibody was added. Protein-G sepharose beads (25 μl of a 50% slurry: Amersham-Pharmacia) were added and samples were incublated overnight at 4°C on a rotating platform. Beads were pelleted and washed three times with NETN buffer (150 mM NaCl, 0.1% NP-40, 10 mM Tris-HCl [pH 8.0], and 1mM EDTA [pH 8.0]). Laemmli sample buffer was added, beads were boiled for four minutes and pelleted by a brief high-speed centifugation. Supernatants containing solubilized proteins were loaded onto 10 or 12% SDS-PAGE gels, electrophoresed and transferred to Immobilon-P. Blots were probed as indicated in each figure.

For IP-kinase assays, 10T1/2 fibroblasts were serum-starved (DMEM containing 0.5-1% FCS) for 16-24 hours. Medium was removed and cells were either refed with medium containing 200 ng/ml TPA (12-*O*-Tetradecanoylphorbol 13-acetate) or exposed to 40 J/m^2 ultraviolet (UV) irradiation (flow rate of 1.5 J/second). Cell extracts were generated using NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. Equal protein concentrations were diluted with NP-40 lysis buffer containing protease inhibitors and 1 μg of antibody and 25 μl of Protein-A or –G (50% slurry) (Amersham-Pharmacia) conjugated sepharose beads were added. IPs were incubated at 4°C overnight on a rotating platform. Beads were collected by centrifugation, washed twice with lysis buffer and twice with kinase buffer (25 mM HEPES [pH 7.6], 20 mM magnesium chloride, 10 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.1 mM sodium vanadate and 2 mM DTT) lacking ATP. Beads were resuspended with an equal volume of kinase buffer containing 50 μM ATP and 5 μCi [γ-^{32}]-ATP. Substrates (2.5 or 5 μg) were added and the reactions were carried out at 30°C for 20 minutes. Reactions were stopped with addition of Laemmlli sample buffer containing DTT and

boiled for four minutes. Beads were pelleted by a brief high-speed centrifugation and supernatants were separated by SDS-PAGE. Gels were stained with Coomassie Blue and destained for a minimum of 12 hours. Gels were dried down and prepared for autoradiography.

GST-Pull Downs

For GST-fusion protein interaction experiments, 250 μg of protein was diluted to a final of 650 μl with NP-40 lysis buffer (containing phosphatase and protease inhibitors) and 5 μg of GST-fusion protein was added. Glutathione beads (Amersham-Pharmacia) were added and samples were incubated overnight at 4°C on a rotating platform. Beads were pelleted, washed and treated as described for immunoprecipitations.

Immunocytochemistry and Immunofluorescence. Cells used for immunocytochemistry were either fixed for six minutes with –20°C methanol and probed with MF20 (Bader *et al.*, 1982) or, cells were fixed for 10 minutes with 4% paraformaldehyde in PBS, washed with three changes of PBS and permeabilized for 5 minutes with 0.3% Triton X-100 in PBS. Plates for immunocytochemistry were blocked for 30 minutes in 5% non-fat-dry-milk dissolved in PBS and incubated for one hour at room temperature with a 1:5 or 1:10 dilution of MF20 (Developmental Studies Hybridoma Bank) antibody supernatant in 5% NFDM. The MF20 monoclonal antibody recognizes essentially all myosin heavy chain isoforms expressed in terminally differentiated sarcomeric muscle-types (Bader *et al.*, 1982). Plates were washed three times with PBS and incubated for one hour at room temperature with a 1:1000 dilution of goat-anti-mouse IgG-horseraddish peroxidase (HRP)-conjugated secondary antibody (BioRad). Plates were washed three times with PBS and incubated with substrate

solution (50 mM Tris [pH 7.6], 0.3% hydrogen peroxide and 6 mg/ml 3,3'-diaminobenzidine tetrahydrochloride) for identification of immune complexes. For immunofluorescence, samples were blocked using an appropriate serum diluted to 10% in PBS for 1 hour at 37°C. Cells were then incubated for 1 hour at room temperature (or overnight at 4°C), with primary antibody diluted in 0.1% bovine serum albumin (0.1% BSA) dissolved in PBS. Cells were washed with three changes of PBS and incubated with secondary antibodies (Sigma) diluted according to manufacturers instruction in 0.1% BSA in PBS. Cells were washed with four changes of PBS and mounted with fluorescence mounting medium (Dako) containing Hoescht 33258 dye for identification of nuclei. All photography was done using Spot Digital camera or a Zeiss Axiovision camera and software.

Computer Software. The following programs were routinely used in these studies: Microsoft Excel for graph preparation, Adobe Photoshop for imaging of microscope images and scanned X-ray films, and Microsoft Powerpoint for figure generation. Any statistics (standard error of the mean calculations) were done using Microsoft Excel.

CHAPTER 3

IMPORTANCE OF MYOD FOR MUSCLE LINEAGE DETERMINATION AND MAINTENANCE

3.1 INTRODUCTION

Transgenic and gene targeting experiments have clearly shown the importance of the myogenic regulatory factors for determination and terminal differentiation of the skeletal muscle lineage (see Introduction section 1.1.4). With the exception of *myogenin*, mice lacking individual MRF genes develop essentially normal muscle. Potential overlapping and redundant functions of the MRFs are suggested by the fact that in the absence of one the expression of another is upregulated and appears to restore normal development of skeletal musculature (see Introduction Section 1.1.4). By contrast, compound mutant mice lacking multiple MRFs demonstrate severe myogenic deficiencies. Importantly, mice lacking both *MyoD* and *Myf5* demonstrate a complete absence of myoblasts and terminally differentiated myofibers (Rudnicki *et al.*, 1993). Together, gene targeting experiments have established a genetic hierarchy with *MyoD* and *Myf5* being necessary for lineage acquistion and function upstream of *myogenin* and *MRF4*, which are required for lineage maintenance and some aspects of terminal differentiation (Megeney and Rudnicki, 1995). While these *in vivo* models have provided tremendous insight into the determination and terminal differentiation of the myogenic lineage, questions persist regarding specific MRF functions, myoblast gene targets and the absolute requirement for *MyoD* and *Myf5* for cell cycle exit and terminal differentiation.

To understand specific molecular details of MRF function, several *in vitro* cell lines exist that permit analyses of gene targets and factors involved in modulating MRF function. In addition, the ability of any of the four factors to convert numerous non-myogenic cells to the myogenic lineage allows for the study of issues pertaining to lineage. Unfortunately, the MRFs form an autoregulatory loop such that expression of one leads to the activation of others making specific determinations of any individual MRF difficult (Thayer *et al.*, 1989; Braun *et al.*, 1989b). For instance, while *in vivo* experiments clearly demonstrate the need for either *MyoD* or *Myf5* for cells to adopt the myogenic fate (Rudnicki *et al.*, 1993), it remains unknown if *myogenin* and *MRF4* are sufficient for faithfull activation of the myogenic program. Similarly, acquisition of the skeletal muscle lineage requires *MyoD* and/or *Myf5* expression but it is currently unknown whether continued expression of either factor is required for complete maintenance of the myogenic fate. Indeed, proliferating myoblasts typically express *MyoD* and/or *Myf5* with *myogenin* and *MRF4* expression typically occurring only after the onset of terminal differentiation (Rudnicki and Jaenisch, 1995; Megeney and Rudnicki, 1995). Loss of myogenicity due to overexpression of activated oncogenes, or prolonged exposure of myoblasts to high concentrations of mitogens, extinguishes the ability of these cells to terminally differentiate due to repression of *MyoD* and *Myf5* expression (see Introduction Section 1.3.3). While the autoregulatory effect of *MyoD* is well-characterized (Thayer *et al.*, 1989), it is currently unknown whether *myogenin* and *MRF4* autoregulate their own expression in the absence of *MyoD* or *Myf5* or, if *myogenin* and *MRF4* are capable of maintaining the myogenic lineage while cells are in a proliferative phase.

To address the role of MyoD in determination and maintenance of the myogenic lineage, immortalized fibroblast cell lines were generated from embryos lacking functional copies of *MyoD* and *Myf5*. These cell lines, termed double knock-out or, DKO, were obtained using a similar protocol as was used to generate the myogenic-permissive fibroblast cell line, C3H10T1/2 (10T1/2) (Reznikoff *et al.*, 1973). Transient transfections with all four myogenic factors revealed these cells are capable of terminally differentiating when any of the four MRFs were expressed suggesting neither *MyoD* and/or *Myf5* are required for myogenesis. Interestingly, DKO cell lines expressing a β-estradiol-activated MyoD-ER fusion protein demonstrated a complete requirement of a functional MyoD molecule for lineage maintanence. These cells competently differentiate in the presence of low-mitogens and β-estradiol in a similar manner as seen with several *in vitro* myoblast cell lines. However, transient exposure of cells to β-estradiol followed by continued passaging in the absence of β-estradiol yielded cells incapable of terminal differentiation and likely reversion to the fibroblastic cell type. This data represents the first direct evidence that the myogenic lineage requires expression of at least one MRF and the myogenic lineage is entirely dependent upon continued expression and appropriate function of at least one myogenic factor.

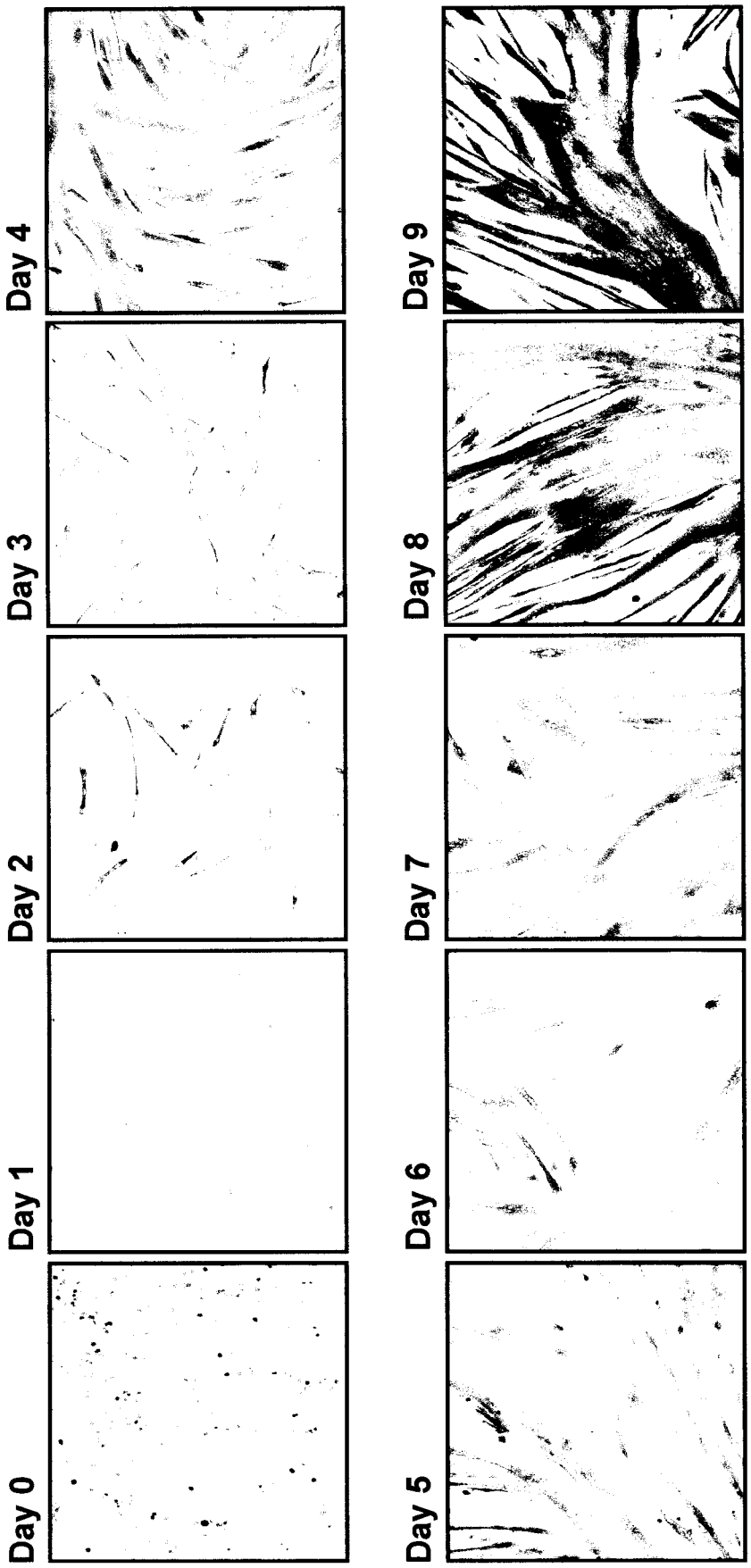
3.2 RESULTS

An important tool for studying myogenesis is the use of myogenic cell lines. Several immortalized cell lines exist and primary satellite cell-derived cultures are relatively easy to establish. In general, determined myoblasts proliferate in high-mitogen containing medium and upon a change to low-mitogen conditions, cells exit the cell cycle, terminally differentiate and fuse to form large multinucleated myotubes. This

characteristic of myoblasts to undergo an essentially normal myogenic program *in vitro* permits detailed study of the molecular events governing myogenesis. In addition, several skeletal muscle-specific markers have been identified and characterized. These markers permit precise characterization of the progress of myogenesis both *in vitro* and *in vivo*. For the purposes of this work, one cell line routinely used was the C2C12 myoblast cell line (Yaffe and Saxel, 1977). These cells proliferate normally in the presence of high mitogens but activate the myogenic program upon refeeding with a low mitogen-containing medium. Terminally differentiated muscle cells can be detected using the MF20 monoclonal antibody (Bader *et al.*, 1982), which recognizes numerous sarcomeric myosin heavy chain (MyHC) isoforms expressed after myoblasts have differentiated (Figure 3.1). It can be seen that MF20 positive cells are detected within 24 hours of refeeding with differentiation medium (DM). As time in DM proceedes, a greater number of cells become MF20 positive and the presence of multinucleated myotubes can be observed (Figure 3.1; compare day2 and day5). The formation of larger myotubes continues and prolonged culturing yields spontaneously contracting myofibers. At the molecular level, proliferating cells express both MyoD and Myf5 but not myogenin or MRF4 (data not shown). Upon a switch to DM, Myf5 levels begin to decrease as cells rapidly induce myogenin expression. MyoD levels typically remain unchanged during differentiation and MRF4 is detected after prolonged culturing (data not shown). While these cells represent an ideal model system for myogenesis, the fact that *MyoD* and *Myf5* are always expressed makes it difficult to study these molecules in isolation. To obtain a clearer understanding of the importance of MyoD in the lineage acquisition and terminal

Figure 3.1: *In vivo* differentiation of the C2C12 myoblast cell line.

C2C12 myoblasts were cultured in growth medium until 70-80% confluency and switched to differentiation medium (DM). Cells were fixed and processed for MF20 immunocytochemistry at the indicated times. The MF20 monoclonal antibody recognizes numerous sarcomeric muscle myosin heavy chain isoforms expressed in terminally differentiated muscle cells. It can be seen that as time in DM progresses, the number of terminally differentiated (MF20 positive; brown staining) cells increases with a concomittant formation of large, multinucleated myotubes as differentiated cells fuse. It should be noted that prolonged culture yields spontaneously contracting muscle fibers. This series demonstrates that terminal differentiation *in vitro* closely follows the events observed *in vivo* and, therefore, represents an excellent model for myogenic study. Cells were counterstained with hematoxylin (purple staining) and photomicrographs were taken using a 10X objective.


Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
Day 8
Day 9
MF20

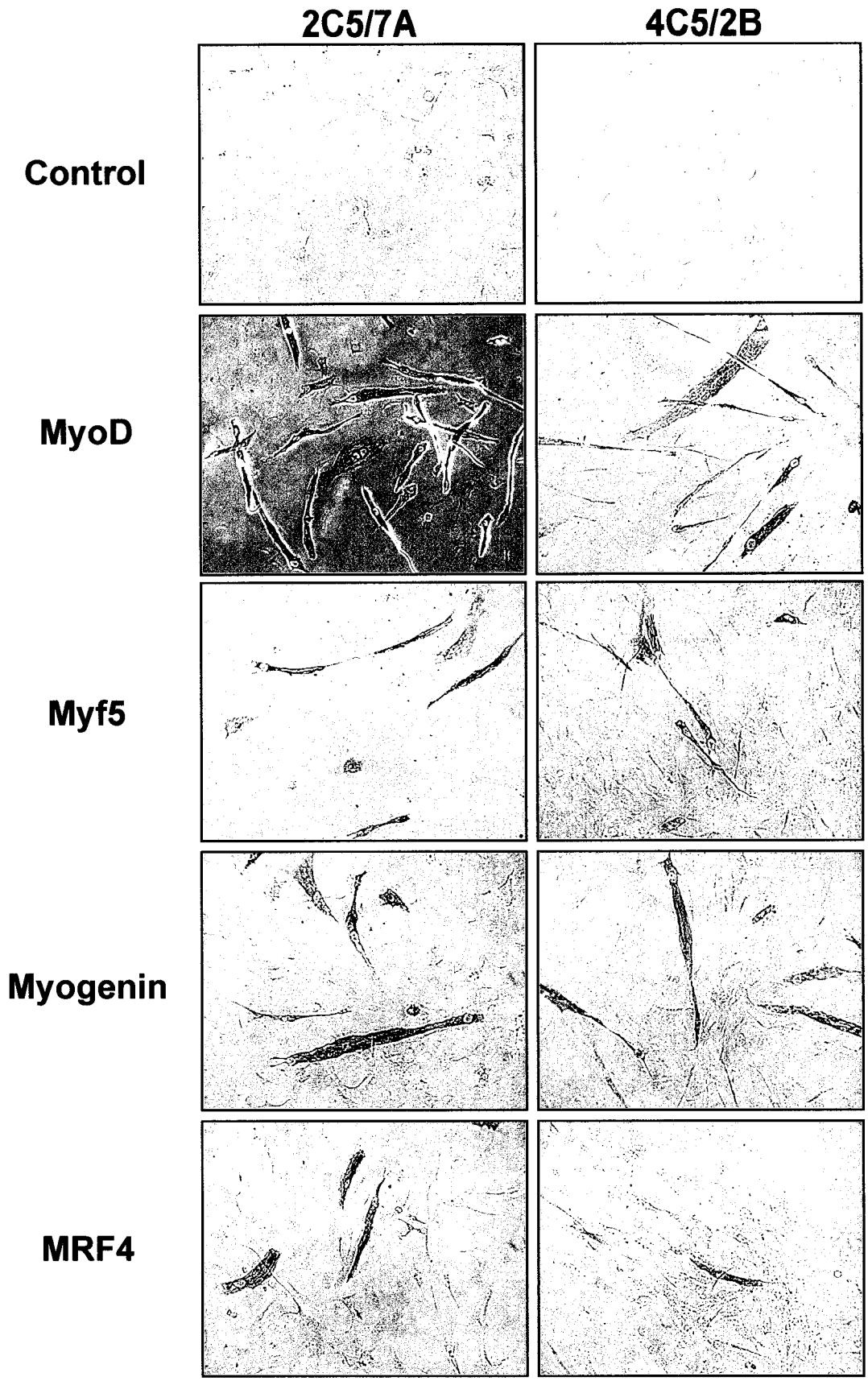
differentiation processes, fibroblast cell lines were derived lacking functional copies of *MyoD* and *Myf5* with the C2C12 cell line representing a control for comparison.

The derivation of fibroblast cell lines lacking functional copies of *MyoD* and *Myf5* is described in the Materials and Methods section. Since mice lacking both *MyoD* and *Myf5* demonstrate a complete absence of myoblasts and muscle fibers (Rudnicki *et al.*, 1993), initial experiments were done to assess if myogenic conversion and terminal differentiation could be induced upon expression of exogenously supplied MRFs in DKO fibroblasts. Due to the fact that mice lacking either *MyoD* or *Myf5* are able to undergo normal myogenesis, the expression of either of these factors should induce the myogenic program in DKO fibroblasts. By contrast, the importance of myogenic determination requiring one of *MyoD* or *Myf5* suggests myogenin and MRF4 may be unable to induce terminal differentiation in the absence of MyoD- or Myf5-mediated lineage determination. Surprisingly, in both cell lines analyzed, all four myogenic factors are capable of inducing skeletal muscle differentiation as assessed by MF20 immunocytochemistry (Figure 3.2). Similar to observations of transfected 10T1/2 fibroblasts, MyoD and myogenin are capable of inducing robust differentiation whereas Myf5 and MRF4 convert lower numbers of cells and the myotubes formed tend to be of smaller size and generally contain a single nucleus. This data indicates that while *in vivo* myogenic lineage determination requires *MyoD* and/or *Myf5* expression, *myogenin* and *MRF4* are able to act independently to activate the myogenic program.

To further examine the importance of MyoD in determining and activating the myogenic lineage, cell lines expressing an inducible form of MyoD were generated. This inducible form of MyoD has the ligand-binding region of the estrogen receptor inserted

Figure 3.2: Induction of terminal differentiation of DKO fibroblasts.

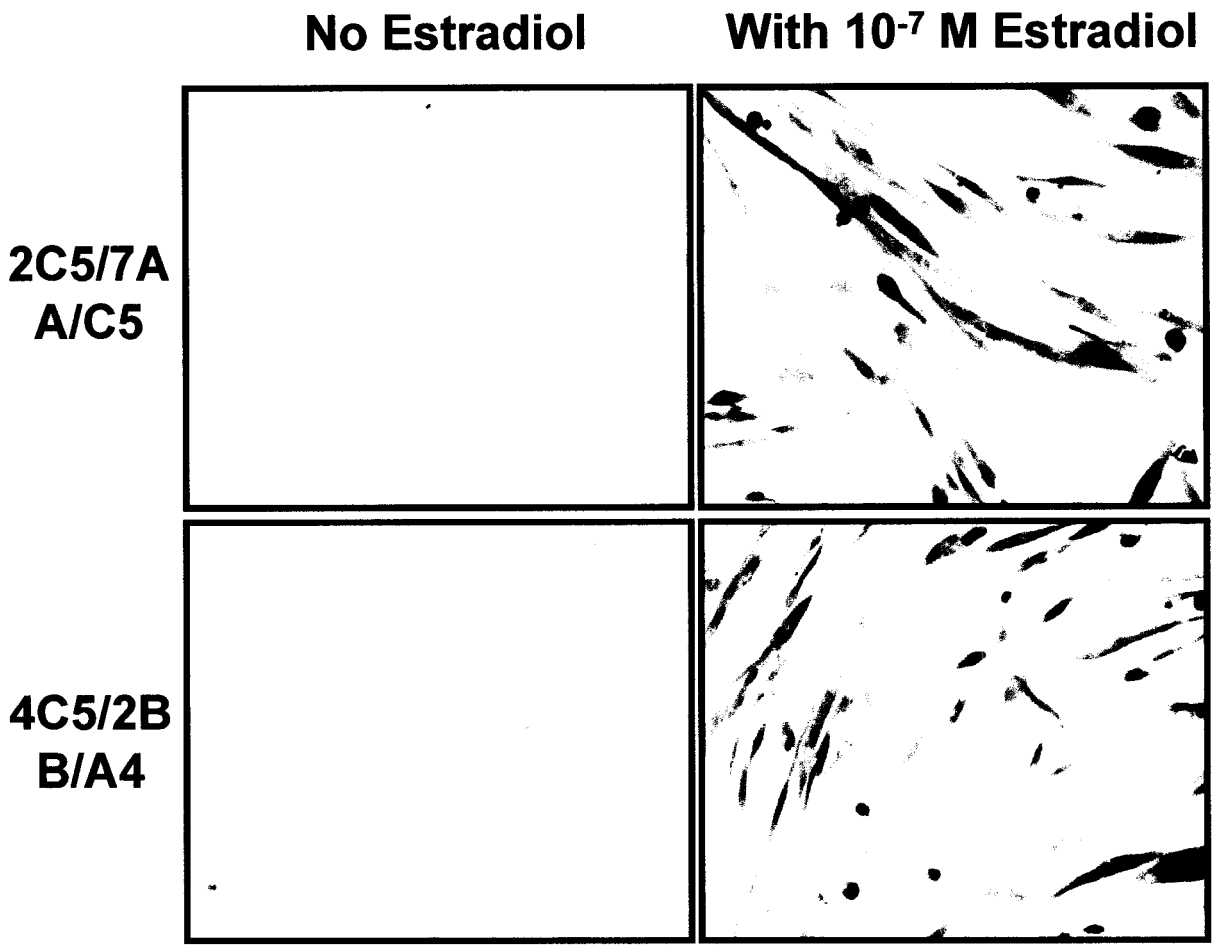
DKO fibroblasts were transfected with 10 μg of an EMSV-control, *MyoD*, *Myf5*, *myogenin* or *MRF4* expression plasmid and transferred to differentiation medium for 5 days. Cells were fixed and terminally differentiated myotubes were detected by MF20 immunocytochemistry (brown staining). All four myogenic factors are capable of inducing a skeletal muscle phenotype in both 2C5/7A and 4C5/2B cell lines. Similar to transfections done in C3H10T1/2 fibroblasts, MyoD and myogenin produce more robust myotubes as compared to Myf5 and MRF4. The ability of myogenin and MRF4 to convert these fibroblasts indicates terminal differentiation does not require either *MyoD* or *Myf5* expression. Photomicrographs were taken using a 20X objective and phase contrast optics.


2C5/7A
4C5/2B
Control
MyoD
Myf5
Myogenin
MRF4
5 Days Differentiation
MF20

downstream of the second helix of MyoD (Hollenberg *et al.*, 1993). Expression of MyoD-ER in the absence of β-estradiol does not lead to activation of the myogenic program as the protein adopts an inactive conformation (Hollenberg *et al.*, 1993; Wyzykowski *et al.*, 2002). In low-mitogen containing medium containing β-estradiol (10^{-7}M) MyoD-ER undergoes a conformational change, dimerizes with an E-protein and activates the myogenic program. It can be seen that in the two cell lines examined exposure of cells to β-estradiol for 2-3 days under proliferating conditions and transfer to DM containing β-estradiol results in the activation of terminal differentiation as assessed by MF20 immunocytochemistry (Figure 3.3). In the absence of β-estradiol exposure, cells are unable to activate myogenesis and remain fibroblastic (Figure 3.3, left panels). It should be noted that cells permitted to proliferate in the absence of β-estradiol and tranferred to DM containing β-estradiol differentiate at a lower efficiency as compared to cells exposed to growth medium containing β-estradiol for 2 or 3 days (data not shown). This suggests that lineage acquisition in the proliferative phase is an important aspect of myogenesis.

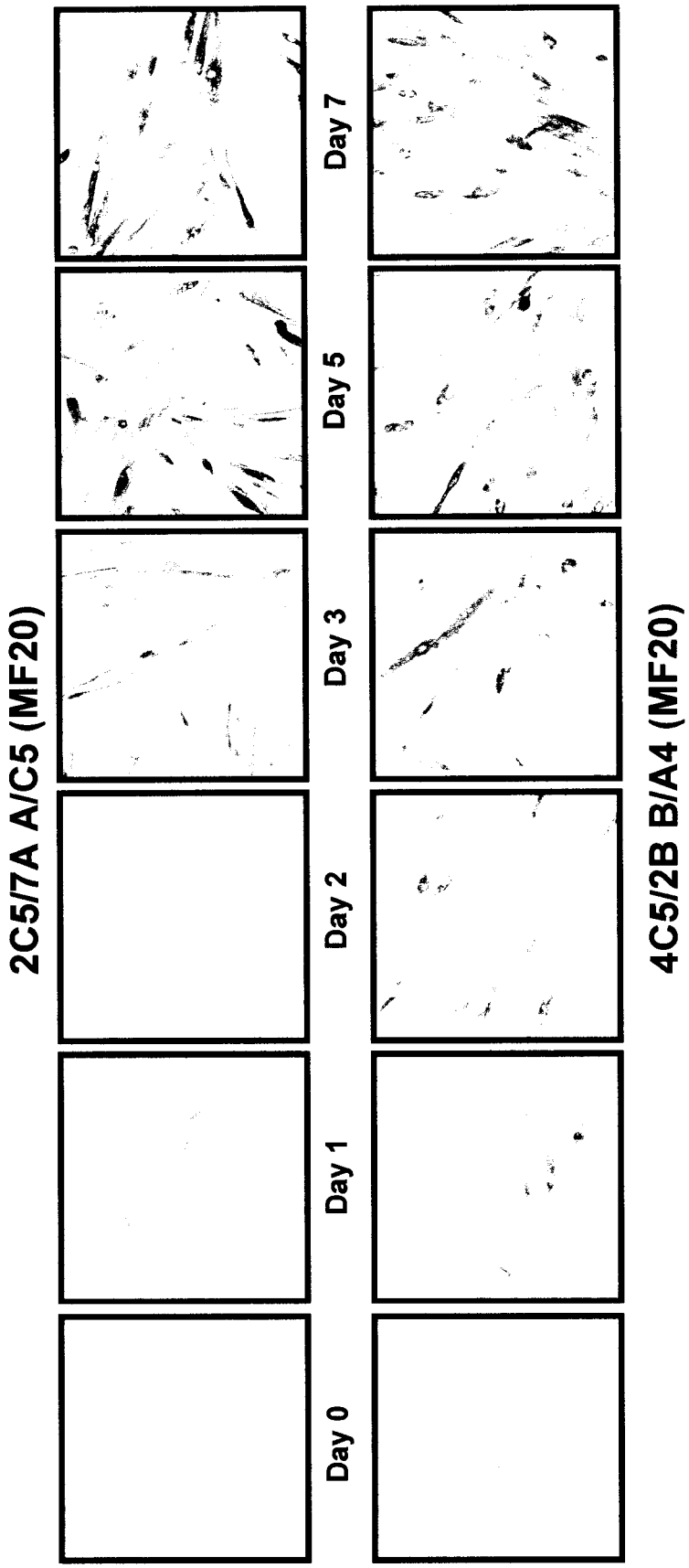
Time course analyses of DKO-MyoD-ER cells revealed that 2C5/7A-A/C5 and 4C5/2B-B/A4 convert to the myogenic lineage and terminally differentiate in a similar manner as observed for C2C12 myoblasts (Figure 3.4). Expsosure of cells to β-estradiol leads to the detection of some MF20-positive cells under proliferation conditions which appears to be dependent upon cell density rather than the cells being unable to proliferate in the presence of a nuclearly-localized MyoD (Figure 3.4, Day 0). Transfer of cells to DM containing β-estradiol results in the progression of terminal differentiation marked by individual cells differentiating and expressing muscle-specific markers. As time in

Figure 3.3: Induction of terminal differentiation of DKO MyoD-ER cell lines. DKO MyoD-ER cells were exposed to growth medium in presence or absence of β-estradiol (10^{-7} M) and transferred to DM with or without β-estradiol for 5 days. Inclusion of β-estradiol yields a functional MyoD-ER molecule, leading to activation of the myogenic program. Terminally differentiated myotubes were detected by MF20 immunocytochemistry (brown staining). Cells not exposed to β-estradiol in either proliferative or low-mitogen conditions do not express markers of terminal differentiation (left panels). Cells exposed to growth medium with β-estradiol for 3 days and transferred to DM containing β-estradiol for 5 days demonstrate numerous MF20-positive myotubes indicating activation of the myogenic program (right panels). Cells were counterstained with hematoxylin (purple staining) to show all nuclei and photomicrographs were taken using a 20X objective.



5 Days Differentiation
MF20

Figure 3.4: Time course terminal differentiation of DKO MyoD-ER cell lines. DKO MyoD-ER cells were passaged and supplemented with 10^{-7} M β-estradiol for 3 days in growth medium. Cells were transferred to differentiation medium containing β-estradiol for the time indicated. Cells were fixed, processed for MF20 immunocytochemistry (brown staining) to detect terminally differentiated myotubes and counterstained with hematoxylin (purple staining) to show all nuclei. In both cell lines examined, the process of differentiation is similar to that observed for established myogenic cell lines, with increases in myotube number and size observed over time as the cells are cultured under low-mitogen conditions. Photomicrographs were taken using a 20X objective.

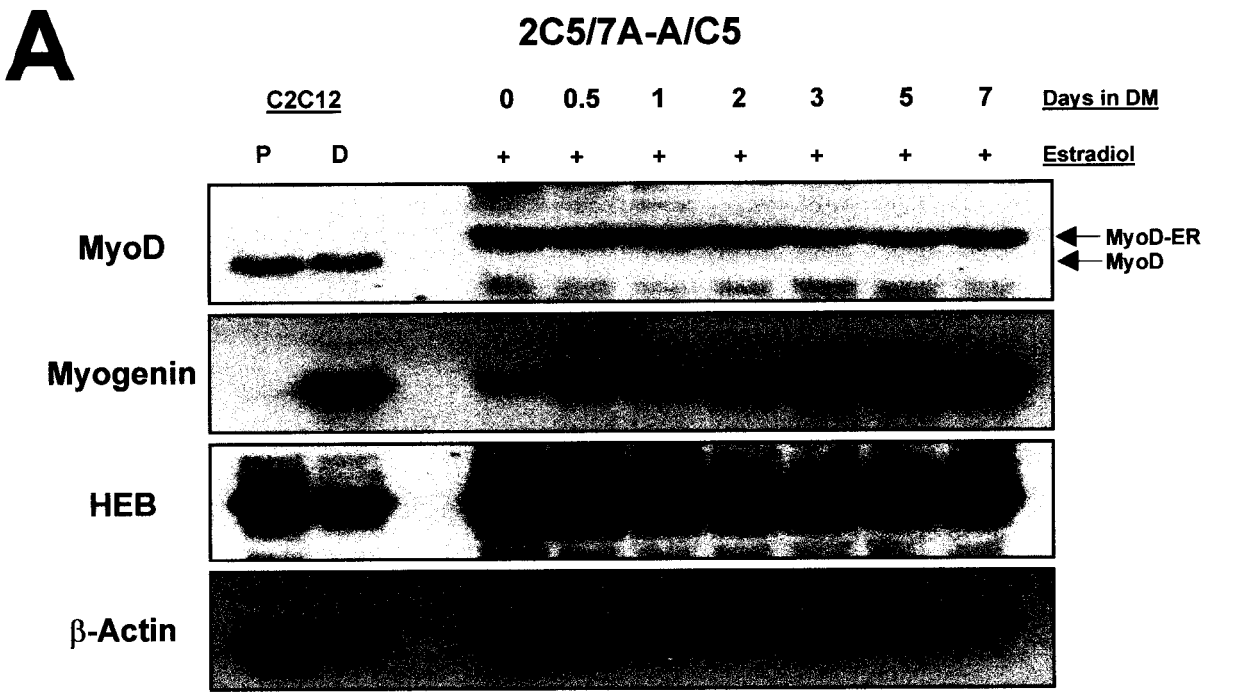

2C5/7A A/C5 (MF20)
4C5/2B B/A4 (MF20)
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7

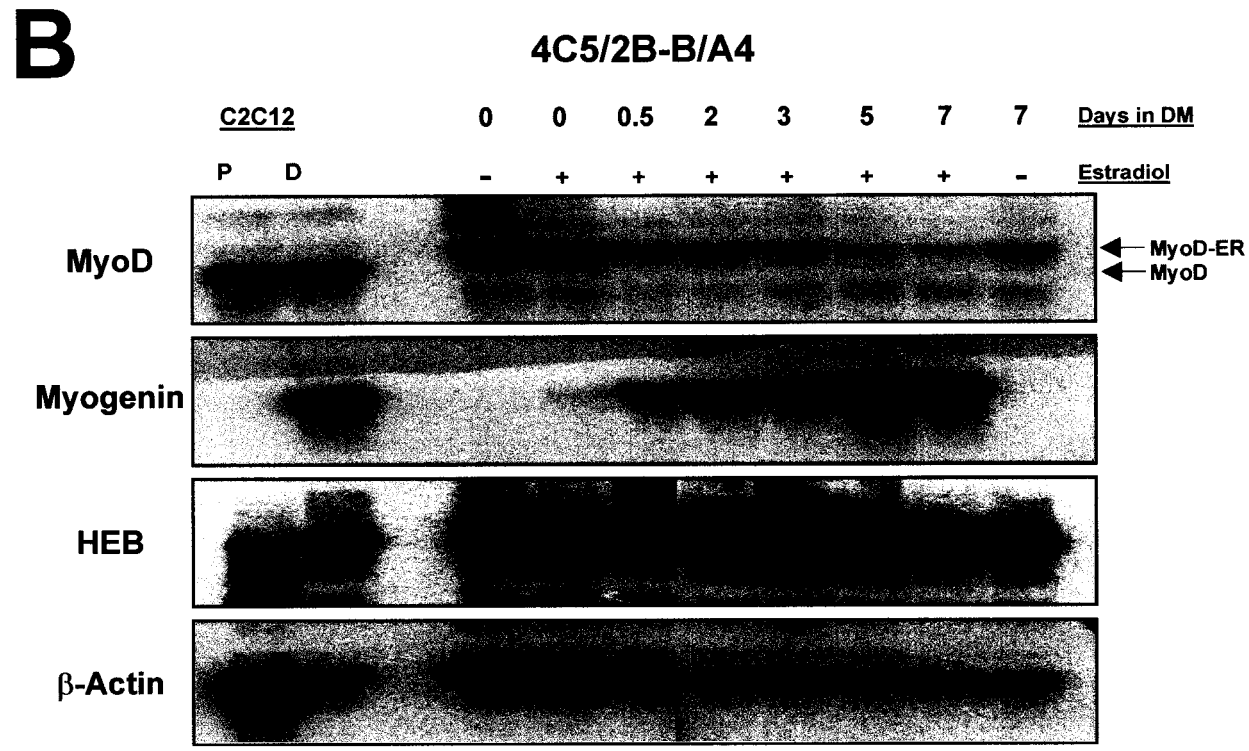
culture progresses, the formation of multinucleated myotubes occurs (Figure 3.4, compare Day 1 to Day 7), reminiscent of that seen with C2C12 myoblasts (Figure 3.1). This data indicates that activation of the MyoD-ER fusion protein is required for terminal differentiation to occur and these cell lines behave in a similar manner as existing *in vitro* culture models.

To confirm that skeletal-muscle specific conversion and terminal differentiation were occuring, Northern blots were done to show the induction of the differentiation-specific gene myogenin (Figure 3.5). In both 2C5/7A-A/C5 and 4C5/2B-B/A4 it can be seen that expression of the MyoD-ER transcript is present in cells under all conditions (Figure 3.5 A and B, upper panels). It should be noted that addition of the ER portion increase the size of the mRNA of MyoD, leading to a decreased mobility (compare C2C12 band with DKO-MyoD-ER bands). The lower, wild-type form of *MyoD* is never detected in these cells confirming the lack of a functional copy of the *MyoD* gene. As seen with MyHC (myosin heavy chain) expression, *myogenin* levels increase during the time in culture under differentiation conditions in the presence of β-estradiol. Importantly, *myogenin* expression is not detected in cells that were under conditions conducive to terminal differentiation but not exposed to β-estradiol (Figure 3.5B, Day 7 no β-estradiol). Induction of *Myf5* does not occur although an aberrant transcript is detected which likely represents a splicing variant of the neomycin resistence gene with *Myf5* sequence (data not shown; (Braun *et al.*, 1992)). As with *MyoD*, the endogenous *Myf5* transcript is never detected in these cells confirming that DKO cells lack a functional copy of the *Myf5* gene. Expression of the E-protein MRF dimerization partner HEB does not vary during the time examined and β-actin represents a loading control

Figure 3.5: Northern blot analysis of DKO MyoD-ER differentiation.

Total RNA was extracted from proliferating (P) C2C12 myoblasts and day 6 differentiated (D) C2C12 myotubes and from DKO MyoD-ER cells during a time course of terminal differentiation and probed as indicated. DKO MyoD-ER cells were cultured in the presence (+) or absence (-) of 10^{-7} M β-estradiol. Northern blots were generated using 15 μg of total RNA in each lane and probed as indicated. (A) 2C5/7A-A/C5 expression shows that the *MyoD* transcript has a higher mobility compared to C2C12 myoblasts due to the addition of the estrogen-receptor ligand-binding region. As with increased MF20 staining, *myogenin* levels increase as cells differentiate indicating they are terminally differentiating along the skeletal muscle pathway. Levels of the dimerization partner *HEB* do not change over time and *β-actin* represents a loading control. (B) 4C5/2B-B/A4 cells show a similar pattern of *myogenin* expression with cells cultured in the absence of β-estradiol unable to induce expression (day 0 and 7 no estradiol). Levels of *HEB* are essentially unchanged and *β-actin* represents a loading control.


A
2C5/7A-A/C5
C2C12
0 0.5 1 2 3 5 7
Days in DM
P D
+ + + + + + +
Estradiol
MyoD
Myogenin
HEB
β-Actin
MyoD-ER
MyoD


B
4C5/2B-B/A4
C2C12
0 0 0.5 2 3 5 7 7
Days in DM
P D
- + + + + + + -
Estradiol
MyoD
Myogenin
HEB
β-Actin
MyoD-ER
MyoD

(Figure 3.5 A and B, lower panels). While *MRF4* is barely detectable in differentiated C2C12 myotubes, expression is either below detection or, does not occur in these cells during the time course examined (data not shown).

Taken together, the DKO fibroblast cell lines clearly show that terminal differentiation can occur in the absence of functional *MyoD* and *Myf5* genes and that terminal differentiation requires a functional MRF to be present in the nucleus. Moreover, time course experiments showed that MyoD-ER cells terminally differentiate in a similar manner as that observed with existing *in vitro* culture models as determined by both morphological (myotube formation) and molecular levels (MyHC and myogenin expression). Therefore, these cells represent a suitable model for studying questions pertaining to lineage acquistion, lineage maintenance and terminal differentiation.

Expression of *MyoD* and/or *Myf5* is associated with determined myoblasts whereas expression of *myogenin* and *MRF4* is observed upon activation of the differentiation program. Moreover, the MRFs form an autoregulatory loop such that expression of one, for instance by transfection into a non-muscle cell type, leads to the expression of the others (Thayer *et al.*, 1989; Braun *et al.*, 1989b). Indeed, stimulation of proliferating MyoD-ER 10T1/2 fibroblasts results in the activation of the endogenous *MyoD* gene, leading to sustained myogenicity upon hormone removal (Hollenberg *et al.*, 1993; Wyzykowski *et al.*, 2002). Moreover, 10T1/2 MyoD-ER cells undergo MyoD-dependent changes in gene expression indicating they represent determined myoblasts (Wyzykowski *et al.*, 2002). To address the role of MyoD in determination and maintenance of the myogenic lineage, DKO-MyoD-ER cells were analyzed for their ability to maintain myogenicity after β-estradiol removal. Unlike 10T1/2 fibroblasts,

DKO-MyoD-ER fibroblasts are unable to autoactivate *MyoD* and/or *Myf5*. Therefore, they represent an ideal system to investigate the requirement for an active MRF in proliferating myogenic cells to maintain the myogenic fate particularly in light of the low levels of myogenin that are detected by Northern blotting of β-estradiol stimulated cells under proliferative conditions.

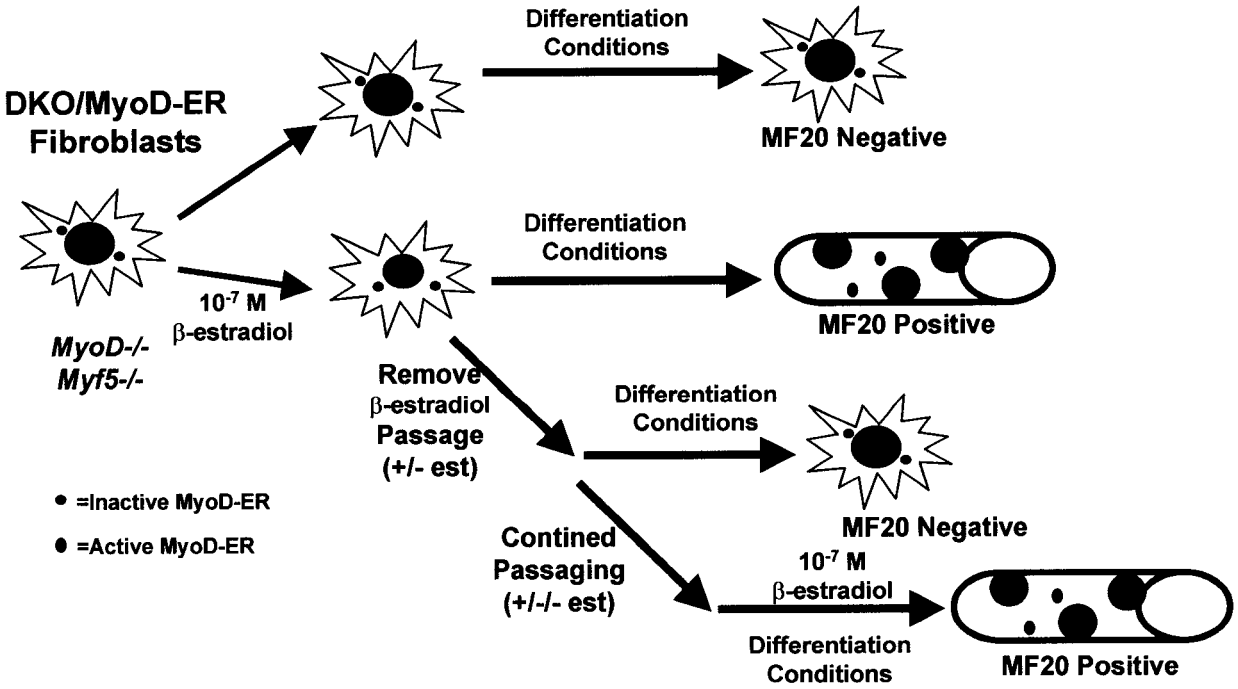
To understand the nature of myogenic determination, DKO-MyoD-ER cells were converted to the myogenic lineage by exposure to β-estradiol under proliferative conditions and subsequently passaged in the absence of β-estradiol to determine if the myogenic fate is maintained (Figure 3.6). The regime these cells were exposed to is shown schematically (Figure 3.6 A). Cells exposed to β-estradiol for three days in growth medium and subsequently passaged in the absence of β-estradiol show a complete inability to terminally differentiate as assessed by MF20 immunohistochemistry (Figure 3.6B, left panels). This suggests these cells have completely lost their myogenic identity. However, cells re-exposed to β-estradiol are capable of terminal differentiation at a similar level as observed in cells exposed for the first time (Figure 3.6B, right panels). Since there is essentially no change in the ability of these cells to differentiate upon re-exposure, it is unlikely that a selection process of cells resistent to MyoD-ER occurred during the initial exposure.

Similar to MF20 staining, Northern blots demonstrate that *myogenin* expression is detected only when cells are exposed to β-estradiol (Figure 3.7). This phenomenon is observed with expression of *myogenin* and it can be seen that cells exposed to β-estradiol and subsequently passaged in the absence do not show a reduction in *MyoD-ER* expression (Figure 3.7). This indicates that while MyoD is capable of inducing the

Figure 3.6: Lineage maintenance requires a functional MyoD molecule.

To assess the requirement of a functional MyoD protein for the maintenance of the myogenic program, cells were cultured in the presence of β-estradiol, with subsequent passages in the absence of β-estradiol with MF20 immunocytochemistry being used to determine myogenic differentiation after 5 days under differentiation conditions. (A) Schematic diagram of the conditions used to assess the ability of DKO MyoD-ER cells to remain myogenic after a transient exposure to β-estradiol and subsequent treatment to assess myogenic lineage maintenance. (B) MF20 immunocytochemistry (brown staining) of DKO MyoD-ER cells under terminal differentiation conditions after one passage in the absence of β-estradiol (left panels) and upon restimulation with β-estradiol after two successive passages in the absence of β-estradiol (right panels). The ability of skeletal muscle specific terminal differentiation to occur requires the presence of a functional MyoD molecule with the myogenic lineage being lost after β-estradiol removal and continued proliferation. This suggests the myogenic lineage can be modulated such that cells exposed to active MRFs do not necessarily remain myogenic. Similar levels of terminal differentiaton in cells re-exposed demonstrates that the loss of myogenic potential is not due to the loss of cells capable of terminally differentiating in the presence of β-estradiol. Cells were counterstained with hematoxylin (purple staining) after immunocytochemistry and photomicrographs were taken with a 20X objective.

A


DKO/MyoD-ER
Fibroblasts
MyoD-/-
Myf5-/-
10-7 M
β-estradiol
Differentiation
Conditions
MF20 Negative
Differentiation
Conditions
MF20 Positive
Remove
β-estradiol
Passage
(+/- est)
Differentiation
Conditions
MF20 Negative
Contined
Passaging
(+/-/- est)
10-7 M
β-estradiol
Differentiation
Conditions
MF20 Positive
● =Inactive MyoD-ER
● =Active MyoD-ER

B

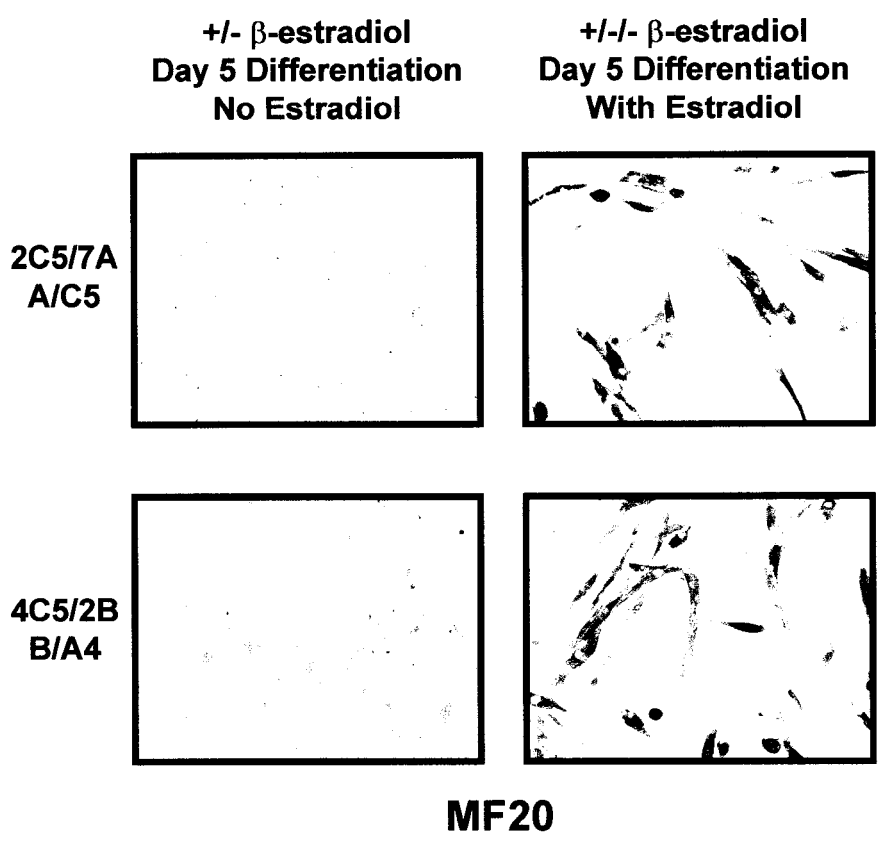
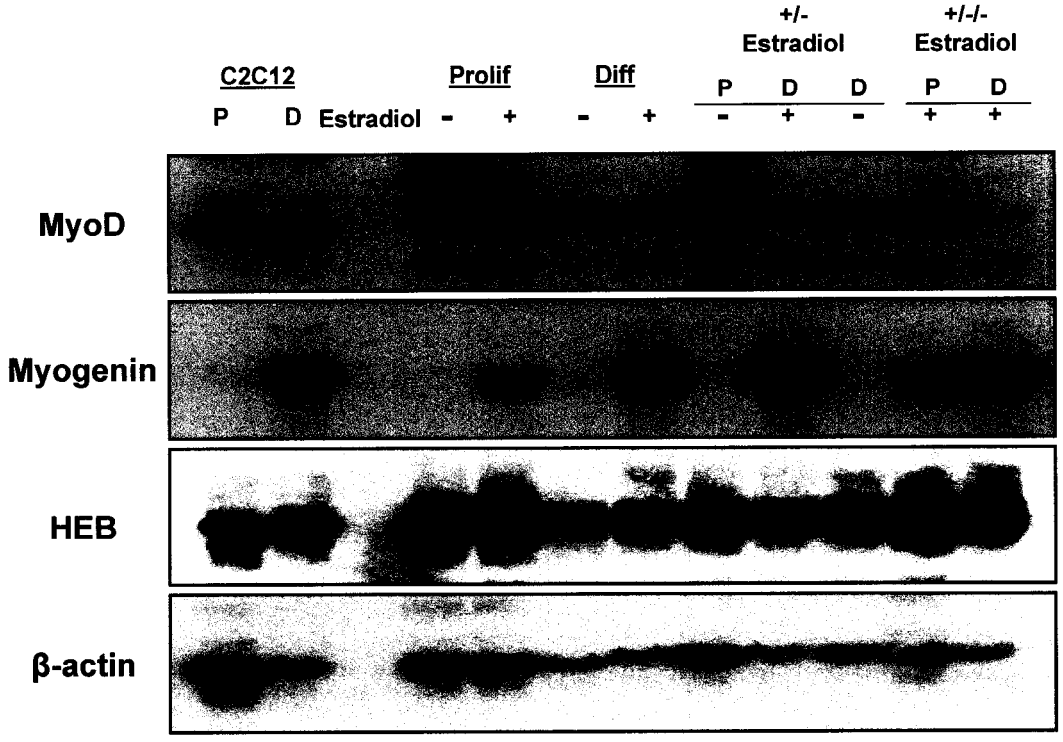

+/- β-estradiol
Day 5 Differentiation
No Estradiol
+/-/- β-estradiol
Day 5 Differentiation
With Estradiol
2C5/7A
A/C5
4C5/2B
B/A4
MF20

Figure 3.7: Northern analysis of DKO MyoD-ER lineage loss.

Total RNA was isolated from proliferating (P) C2C12 myoblasts or day 6 differentiated C2C12 (D) myotubes and from DKO MyoD-ER cells under growth or differentiation conditions in the presence (+) or absence (-) of 10^{-7} M β-estradiol. Northern blots were generated using 15 μg of total RNA in each lane and probed as indicated. Cells that were singly passaged after β-estradiol exposure and analyzed for their ability to terminally differentiate in the absence of β-estradiol are denoted as +/- estradiol. Cells passaged twice after β-estradiol exposure and re-exposed are denoted as +/-/- estradiol. The dependence of myogenin expression on β-estradiol stimulation shows that the skeletal muscle lineage requires a functional MyoD molecule. Re-expression of myogenin upon restimulation with β-estradiol indicates that the cells present do not represent a population of cells originally insensitive to MyoD-ER and that the lineage of these cells is completely reversible. Levels of HEB remain unchanged and β-actin represents a loading control.


+/-
Estradiol
+/-/-
Estradiol
C2C12
Prolif
Diff
P D Estradiol - + - + P D D P D
- + - - + +
MyoD
Myogenin
HEB
β-actin

myogenic lineage, it must be in an active conformation for this process to occur. However, the exposure of cells to at least one round of cell division is important as cells exposed to β-estradiol in growth medium differentiate, albeit at a markedly reduced rate, when transferred to DM lacking β-estradiol (data not shown).

Taken together, this data represents the first clear evidence that MyoD, or expression of the other MRFs and in an active conformation, is absolutely required for myogenic lineage determination and terminal differentiation. Moreover, this data suggests the cells require sustained MRF expression to maintain the myogenic lineage with loss of expression leading to reversion of the cells to a pre-myogenic state similar to lineage loss due to exposure of myoblasts to high levels of mitogens or, constitutive expression of activated oncogenes.

3.3 DISCUSSION

Important to myogenic lineage determination *in vivo* is the expression of *MyoD* and/or *Myf5* during development. In the absence of expression, determined myoblasts and muscle fibers are unable to develop (Rudnicki *et al.*, 1993). In this chapter, data was presented that clearly shows that while the determination of the myogenic lineage *in vivo* requires *MyoD* or *Myf5* expression, their absence does not preclude an inability of cells to adopt a myogenic fate. Indeed, expression of myogenin or MRF4 in fibroblast cell lines lacking functional copies of *MyoD* and *Myf5* sufficiently activated the myogenic program, leading to terminal differentiation and the expression of skeletal muscle-specific markers. DKO fibroblasts stably expressing an inducible form of MyoD demonstrated that maintenance of the myogenic lineage requires sustained expression of a functional MRF, with loss of expression/function leading to lineage reversal. This data

supports the hypothesis that acquisition of the myogenic lineage and maintenance of myoblast identity require sustained expression of *MyoD* and/or *Myf5*.

The cells detailed in this chapter represent a unique model system for the study of factors involved in several aspects of myogenesis. One unique characteristic of these cells is that they are unable to activate endogenous *MyoD* and *Myf5* genes, thus providing a unique opportunity for the analysis of MyoD function both in proliferating myoblasts and differentiated myotubes. Previous studies using 10T1/2 fibroblasts stably expressing the MyoD-ER fusion protein have provided insight into aspects of lineage acquisition and maintenance (Hollenberg *et al.*, 1993; Gerber *et al.*, 1997; Wyzykowski *et al.*, 2002), MyoD-mediated chromatin remodelling (Gerber *et al.*, 1997) and changes in gene expression patterns of the proliferative myoblast (Wyzykowski *et al.*, 2002). Data obtained from research examining MyoD-dependent gene activation using the DKO fibroblast system has shown that MyoD activates numerous gene subsets upon the initiation of terminal differentiation (Bergstrom *et al.*, 2002). This study demonstrated that MyoD regulates a wide-array of gene subsets, defining a series of myogenic subprograms. Importantly, DKO MyoD-ER cells pre-treated with cyclohexamide, a protein synthesis inhibtor, or the p38 kinase inhibitor SB 203580 show that MyoD-dependent gene subsets are differentially activated during terminal differentiation. This research permitted the conclusion that while the expression of all genes involved with terminal differentiation are required for proper myogenesis, several intracellular systems exist that modulate the expression of very specific gene sets, forming an intricate network of MyoD-regulated myogenic subprograms (Bergstrom *et al.*, 2002).

An important aspect of terminal differentiation is the requirement of myoblasts to exit the cell cycle (see Introduction Section 1.3.1). A great deal of research analyzing this aspect of terminal differentiation shows a requirement of the retinoblastoma (Rb) protein in preventing the reentry of differentiated muscle cells into the cell cycle (Schneider *et al.*, 1994; Mal *et al.*, 2000) and preventing apoptosis (Wang *et al.*, 1997b). While early reports suggested a direct interaction of MyoD with Rb (Gu *et al.*, 1993), more recent studies suggest this interaction is not found in cells (Zhang *et al.*, 1999c) and that the MEF2 family of transcription factors are vital for cooperating with Rb to repress cell cycle reentry (Novitch *et al.*, 1999). The ability to 'deactivate' MyoD in DKO-MyoD-ER fibroblasts by removing β-estradiol permits the study of how terminal differentiation and cell cycle reentry are regulated after the onset of terminal differentiation. Indeed, preliminary data obtained with the DKO-MyoD-ER fibroblasts suggest that removal of a functional MyoD after the onset of differentation does not affect the terminally differentiated phenotype or lead to cell cycle reentry (data not shown). This suggests that terminal differentiation is maintained in the absence of MyoD and Myf5 and, therefore, these factors are dispensible for later stages of myogenesis. This is supported by the observation that myogenin and MRF4 are capable of inducing terminal differentiation in the absence of *MyoD* and *Myf5* expression (Figure 3.2). Future experiments will analyze the expression patterns of the Rb family of proteins and the expression profile of the MEF2 proteins. It is likely that upon *myogenin* expression, an autoregulatory loop is established between myogenin and MEF2 proteins to maintain the differentiated state as has been previously suggested (Naidu *et al.*, 1995; Edmondson *et al.*, 1994).

Another important aspect of myogenesis is the maintenance of the myogenic identity of proliferating myoblasts. Surprisingly, little information exists regarding MyoD/Myf5 gene targets in myoblasts and the nature of how the myogenic identity is maintained in proliferating myoblasts. Recent studies using the 10T1/2 MyoD-ER cell line suggest two markers, *Id3* and *NP1*, that are preferentially activated by MyoD and may represent myoblast-specific gene targets (Wyzykowski *et al.*, 2002). Due to the ability of DKO MyoD-ER cells to lose their myogenic identity, these cells represent an ideal system with which to isolate and characterize putative MyoD gene targets that are specifically activated in myoblasts. Current experiments are using cDNA arrays to compare the gene expression profiles of DKO cells after infection with lentivirus and expression of *MyoD* or *Myf5*. Furthermore, a dual-selection gene trap vector is being employed in the DKO MyoD-ER cell lines. Gene trap vectors have been successfully used in the past to identify early embryonically expressed genes in embryonic stem cells (Friedrich and Soriano, 1991; Chen *et al.*, 1994) and to obtain genes regulated by growth factor stimulation (Akiyama *et al.*, 2000). Since specific genetic expression profiles are clearly evident upon activation of the MyoD-ER protein in 10T1/2 fibroblasts (Wyzykowski *et al.*, 2002), a dual-selection gene-trap system, similar to that described elsewhere (Medico *et al.*, 2001), will permit the rapid and specific isolation of MyoD-dependent genes. One advantage for using a gene-trap, as a complementary technique with cDNA arrays, is that one is able to efficiently detect genes with inducible expression, even those demonstrating extremely low expression levels (Akiyama *et al.*, 2000; Medico *et al.*, 2001). Similarly, the ability of these cells to be manipulated will provide an excellent model for analyzing critical aspects of lineage acquisition and the

nature of how MyoD maintains the myogenic lineage under conditions of high mitogenic signaling. In addition, the ability to shut off MyoD function after the onset of differentiation will permit the study of the molecular aspects required for maintenance of the terminally differentiated state.

CHAPTER 4

LEVEL OF MRF REGULATION BY MEK1

4.1 INTRODUCTION

Initiation of the myogenic program represents a transition from a proliferative, myoblast phase, to a terminally differentiated, myotube phase in which cells have permanently withdrawn from the cell cycle. While proliferating myoblasts express *MyoD* and/or *Myf5*, mitogens and activated oncogenes repress cell cycle exit, MRF-mediated gene expression and terminal differentiation (for review see Perry and Rudnicki, 2000). While it is clear that stimulation of myoblasts with several growth factors inhibits terminal differentiation, in many cases the precise underlying molecular mechanisms remain unclear. Indeed, elucidation of intracellular signal transduction pathways and cloning of the constituent components, has lead to a great deal of research examining these pathways and their role in regulating the MRFs and myogenesis.

Peptide growth factors, cytokines and stress activate a series of intracellular signal transduction pathways. These mitogen-activated protein kinase (MAPK) pathways involve sequential phosphorylation of kinase intermediates and culminate in the alteration of transcription factor activity (for review see Chen *et al.*, 2001). In general, proliferative signals that are initiated by peptide growth factors, such as basic fibroblast growth factor (bFGF), activate the Ras-Raf-MEK-ERK pathway leading cell growth and division. Indeed, the importance of these kinases in cell proliferation is evident in light of the fact that overexpression of activated mutant versions of many of the MAPK components leads to unregulated proliferation and cellular transformation (Chen *et al.*, 2001). Of interest, MAPK activation leads to upregulation of cyclin D1 and cell cycle progression (Jones

and Kazlauskas, 2001). Since cyclin D1 overexpression has been shown to inhibit the myogenic program, this may represent an important link to the nature of growth factor regulation of terminal differentiation (Rao *et al.*, 1994; Skapek *et al.*, 1995). Phosphorylation of serine 200 in the C-terminal portion of MyoD by cyclin/cdk1/2 complexes targets MyoD for ubiquitin-mediated degradation (Kitzmann *et al.*, 1999; Reynaud *et al.*, 1999; Song *et al.*, 1998; Tintignac *et al.*, 2000) whereas, a region of MyoD within this same region has been shown to interfere with cyclin/cdk activity and cell cycle progression (Zhang *et al.*, 1999c; Zhang *et al.*, 1999b). This demonstrates a very complex regulatory network involving the extracellular signaling, cell cycle protein complexes and the initiation of the terminal differentiation program.

Specific studies addressing the involvement of the mitogen-activated protein kinase (MAPK) pathways suggest both positive and negative effects on myogenesis. For example, upregulation of the MAPK phosphatase MPK1 at the onset of differentiation is responsible for a subsequent decrease in MAPK activity (Bennett and Tonks, 1997). Moreover, expression of a dominant inhibitory mutant of MEK1 inhibits the negative effects of growth factors on differentiation (Weyman and Wolfman, 1998). Overexpression of the MAPK upstream activator c-Raf inhibits terminal differentiation of myoblasts (Dorman and Johnson, 1999). Other reports suggest that overexpression of an activated form of MEK1 does not inhibit differentiation (Ramrocki *et al.*, 1997) and that myogenic differentiation requires an increase in MAPK activity (Gredinger *et al.*, 1998).

To address the role of the MAPK pathway in regulating myogenesis, experiments were undertaken to closely examine the role of the ERK-upstream activator MEK1. Using reporter and myogenic conversion assays, the data indicates that MAPK signalling

negatively regulates MRF-dependent gene transcription while not affecting subcellular localization or stability of MyoD. Furthermore, the activated MEK1 mutant used for these studies resides in both nuclear and cytoplasmic compartments suggesting subcellular localization is very important for regulating MRF-mediated transcriptional activity. Interestingly, data showing alterations in the availability of a MRF dimerization partner during the cell cycle suggests an important level of regulation at a critical stage of the myoblast cell division cycle. This represents a potentially important and novel mechanism for regulating cell cycle withdrawal and terminal differentiation.

4.2 RESULTS

Effect of MAPK signaling on MRF-mediated gene expression and differentiation

The ability of growth factors, such as FGF, to repress skeletal muscle differentiation suggests that the signaling pathways regulated by these factors are important for modulating MRF transcriptional activity. To specifically address the role of the MAPK pathway in terminal differentiation, cotransfection experiments were performed using mutant versions of MEK1 and MRF transcriptional activity was assessed by their ability to transactivate skeletal muscle-specific reporter vectors (Figure 4.1). For these experiments, two reporter vectors were chosen. The 4RtkCAT reporter contains four-tandem myogenic E-box motifs, derived from the right-hand MLC enhancer element, upstream of the thymidine kinase minimal promoter (Weintraub *et al.*, 1991). To simulate endogenous gene activation, the MLC-CAT reporter vector (Grieshammer *et al.*, 1992), which contains the promoter and enhancer elements from the myosin light chain 1/3 locus driving CAT expression, was chosen. The MEK1 mutants used for these studies have been described elsewhere (Mansour *et al.*, 1994) and

represent the full-length wild-type (wt MEK1), a dominant negative (DN MEK1) form with a K97M mutation that abolishes kinase activity, and an activated (Act MEK1) version that has an amino-terminal deletion (amino acids 32-51) and two activating mutations S218D/S222E, that simulate the charge created by addition of a phosphate group to serine. All three MEK1 mutants are easily identified due to fusion of the haemagglutin (HA)-tag to the amino-terminus.

It can be seen that the level of MRF-mediated transcriptional activity is dramatically reduced when the constitutively activated form of MEK1 is coexpressed (Figure 4.1). In the case of the engineered 4RtkCAT vector, neither wild-type or dominant negative forms of MEK1 exerted any effect on MRF-mediated transcription (Figure 4.1A). By contrast, wt MEK1 exerts a modest negative effect on the ability of Myf5 and myogenin to activate the MLC-CAT reporter (Figure 4.1B). Similarly, MyoD, and to a lesser extent Myf5, are positively influenced by the coexpression of the DN MEK1 mutant (Figure 4.1B). As seen with the 4RtkCAT reporter, coexpression of the activated form of MEK1 dramatically reduces the transcriptional activity of all four factors. Importantly, these effects are not directed at regulating the EMSV-based MRF expression plasmids as coexpression of MyoD with MEK1 mutants does not affect the expression of an EMSV-CAT reporter vector (Figure 4.2).

One important aspect of MRF expression in non-muscle cell types is the ability of these factors to convert cells to the myogenic lineage (Weintraub *et al.*, 1989; Choi *et al.*, 1990). To assess the ability of the MRFs to convert cells to the myogenic lineage, 10T1/2 cells cotransfected with MyoD and the mutant forms of MEK1 were examined for expression of terminal differentiation-specific markers and whether the stability of

Figure 4.1: Effect of activated MEK1 on MRF-mediated gene expression.

To assess the effect of the MAPK pathway on muscle-specific gene activation, C3H10T1/2 fibroblasts were cotransfected with plasmids encoding the myogenic factors, mutant forms of the ERK-upstream activator MEK1 and 4RtkCAT (A) or MLC-CAT (B) reporter vectors. Cells were transfected as indicated, harvested after 48 hours in differentiation medium and CAT assays were performed. In all cases, coexpression of activated MEK1 with any of the myogenic factors leads to a dramatic reduction in the ability of the MRFs to transactivate either reporter vectors. While wild-type and dominant negative forms of MEK1 do not exert any influence on MRF transactivation of the 4RtkCAT reporter, they do moderately affect MRF-mediated transactivation on the MLC-CAT reporter vector. The bars represent the mean and the error bars represent the standard error of the mean (+/- SEM; n=9).

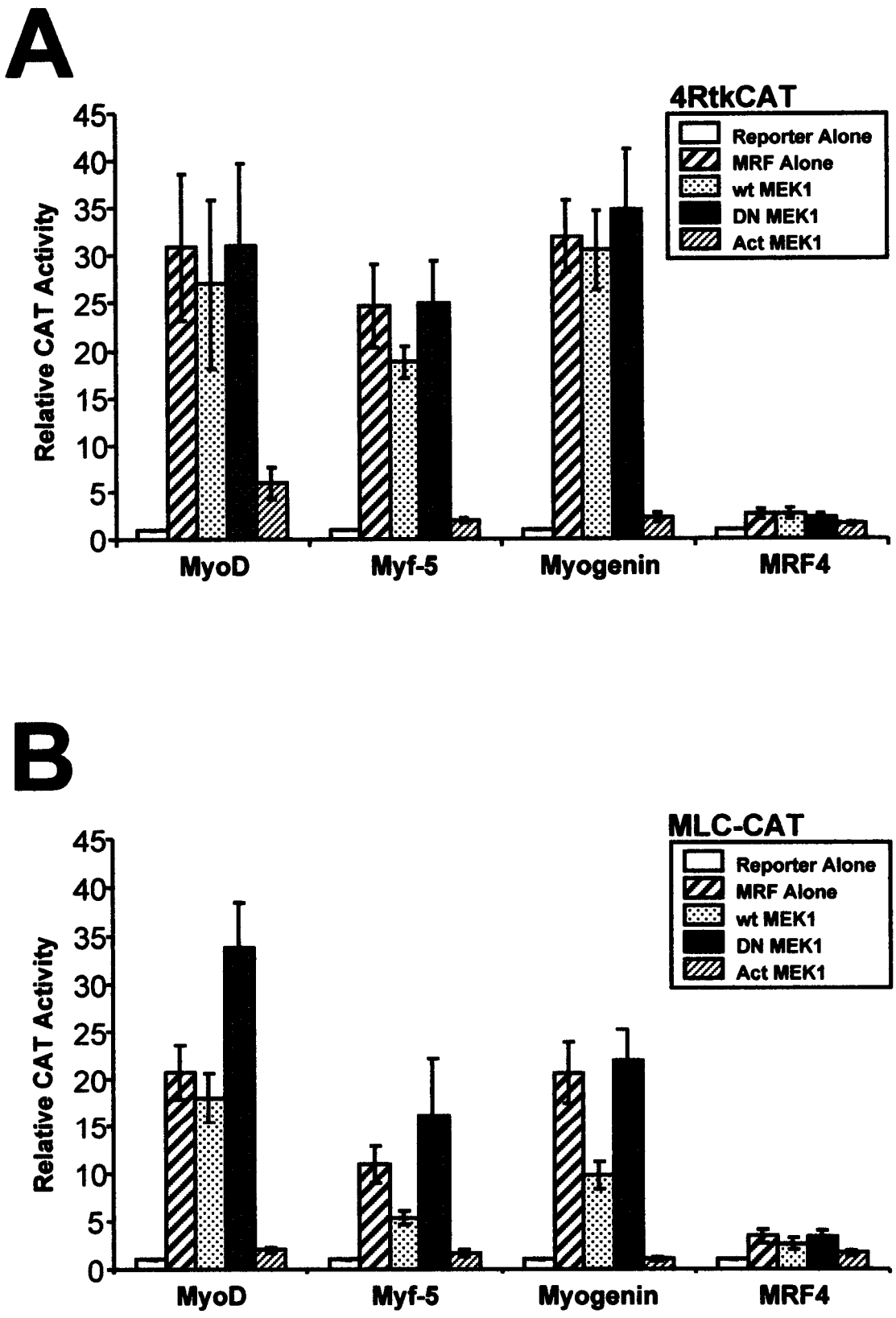
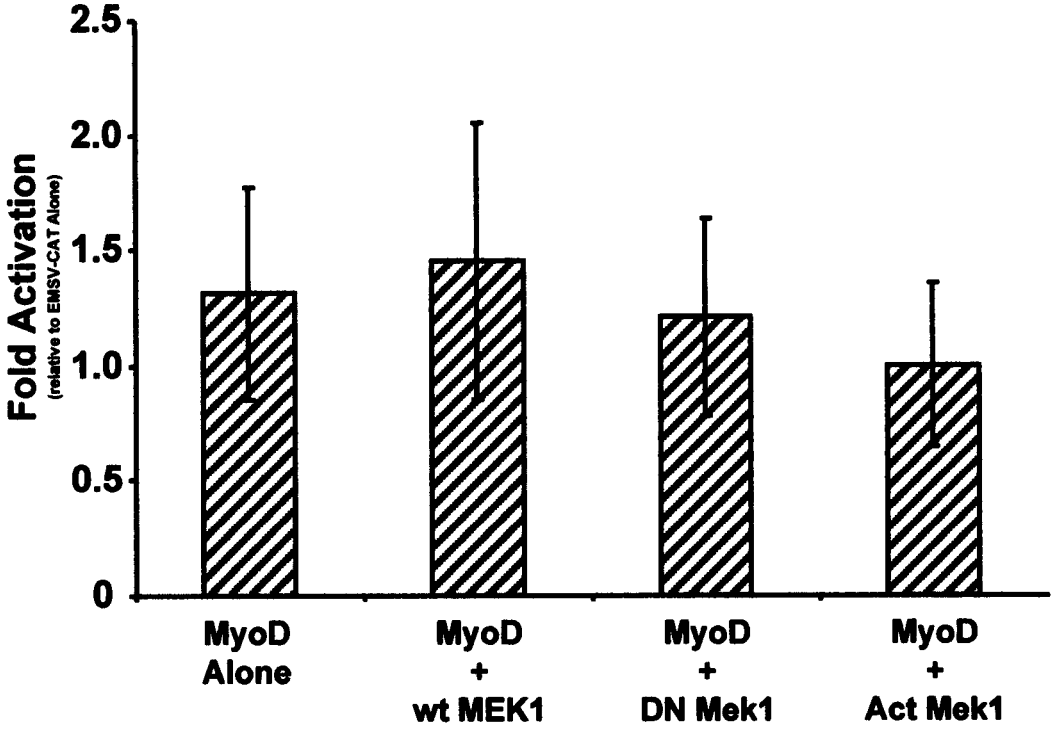

A
4RtkCAT
Reporter Alone
MRF Alone
wt MEK1
DN MEK1
Act MEK1
Relative CAT Activity
0
5
10
15
20
25
30
35
40
45
MyoD
Myf-5
Myogenin
MRF4
B
MLC-CAT
Reporter Alone
MRF Alone
wt MEK1
DN MEK1
Act MEK1
Relative CAT Activity
0
5
10
15
20
25
30
35
40
45
MyoD
Myf-5
Myogenin
MRF4

Figure 4.2: Reduction of MRF-mediated gene expression is not due to MAPK-mediated effects on effector plasmid expression.

To control for the potential effects of MAPK signaling on the expression from the EMSV-MRF expression plasmids, 10T1/2 fibroblasts were cotransfected with MyoD, MEK1 mutants and an EMSV-CAT reporter vector. Cells were harvested after 48 hours in differentiation medium and CAT assays were performed. Values were calculated as fold activation as compared to EMSV-CAT cotransfected with MRF and MEK1 promoter controls. It can be seen that expression levels of the EMSV-based CAT vector is essentially unchanged. This suggests the effects observed on MRF-mediated gene activation are due to specific effects on myogenic factor activity. Bars represent the mean and error bars the standard error of the mean (+/- SEM; n=6)


Fold Activation
(relative to EMSV-CAT Alone)
2.5
2.0
1.5
1.0
0.5
0
MyoD Alone
MyoD + wt MEK1
MyoD + DN Mek1
MyoD + Act Mek1

MyoD was regulated by MEK1 coexpression (Figure 4.3). Transfection of MyoD alone or, in the presence of wt MEK1 or DN MEK1 permit lineage specification and terminal differentiation as assessed by myotube formation and MF20 immunocytochemistry (Figure 4.3A; black arrows). However, as observed with reporter assays, the ability of MyoD to activate the myogenic program in 10T1/2 cells is dramatically reduced by the presence of activated MEK1, as seen by smaller myotubes and substantially fewer MF20-postive cells (Figure 4.3A; black arrows). Importantly, the steady-state levels of MyoD were unaffected by the cotransfection of MEK1 mutants suggesting protein stability is not involved in MEK1 regulation of MyoD function (Figure 4.3B). Similarly, expression levels of MEK1 mutants, as detected by anti-HA immunoblotting, demonstrate essentially equivalent expression levels and there is no change in expression of the MRF dimerization partner, HEB (Figure 4.3B). By contrast, expression of myogenin, a marker of terminal differentiation, is reduced in the presence of activated MEK1 confirming that activation of the myogenic program is impaired in these cells (Figure 4.3B; compare lanes 2-4 with 5). Similarly, the other three MRFs were impaired in their ability to convert 10T1/2 fibroblasts to the myogenic lineage when coexpressed with activated MEK1 (data not shown).

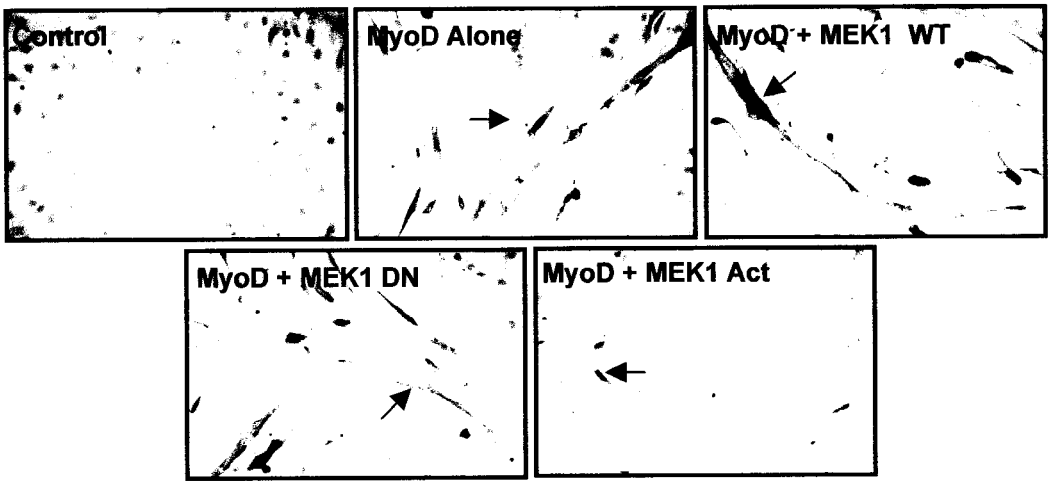
To further analyze the regulation of MRF-mediated gene activation, C2C12 myoblasts were transfected with MEK1 mutants and a constitutively expressed GFP (green fluorescent protein) reporter vector and the effect on myotube formation was assessed (Figure 4.4). C2C12 myoblasts transfected with GFP alone or with wild-type or dominant negative forms of MEK1 form robust, GFP-positive myotubes (Figure 4.4; red arrows). However, as observed in 10T1/2 cotranfections, expression of the activated

Figure 4.3: Activated MEK1 inhibits MRF-mediated myogenic conversion but does not affect MyoD stability.

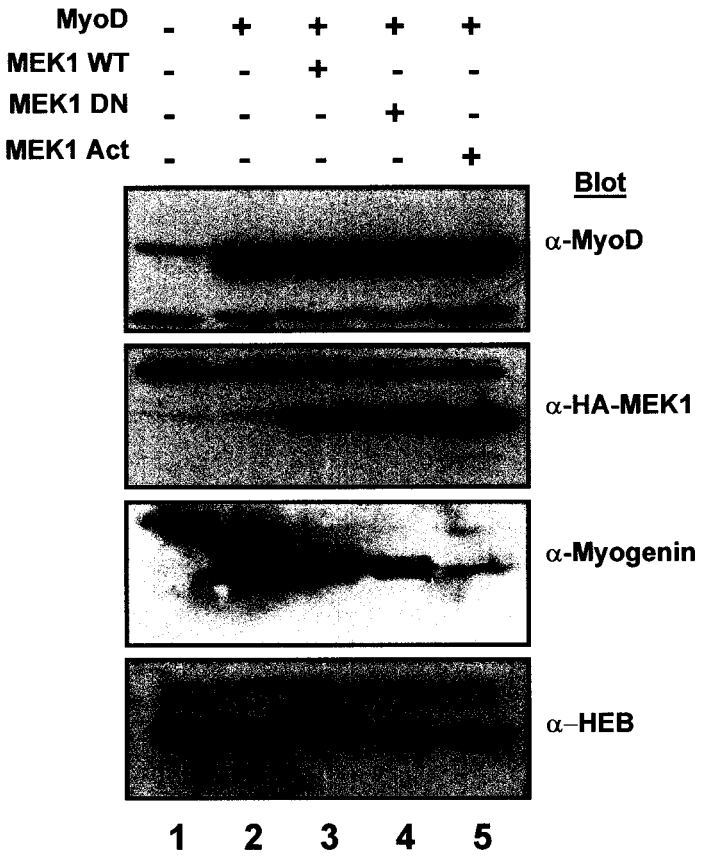
(A) C3H10T1/2 fibroblasts were transfected with the indicated plasmids. Conversion to the myogenic lineage was assessed by myotube formation (black arrows) and MF20 immunocytochemistry (brown staining) after 48 hours in differentiation medium. As seen with MRF-mediated gene expression, the ability of MyoD to myogenically convert 10T1/2 fibroblasts and induce terminal differentiation is greatly reduced in the presence of activated MEK1. Cells were counterstained with hematoxylin (purple staining) and photomicrographs were taken using a 20X objective.

(B) Immunoblotting for transfected gene products and markers of terminal differentiation demonstrate that as with MF20 immunocytochemistry, reduced myogenin levels are observed in extracts prepared from cells transfected with MyoD and activated MEK1. Levels of HA-tagged MEK1 mutants are similar and the reduction in myogenesis is not due to a destabilization of MyoD protein as steady-state levels remain unchanged irrespective of the MEK1 mutant coexpressed. Similarly, levels of the E-protein dimerization partner, HEB, are unchanged in cells transfected with MyoD and mutant MEK1 vectors. Cells were harvested after 48 hours in differentiation medium. Immunoblots were produced using 25 μg of protein extract and probed as indicated.

A


Control
MyoD Alone
MyoD + MEK1 WT
MyoD + MEK1 DN
MyoD + MEK1 Act

B


MyoD - + + + +
MEK1 WT - - + - -
MEK1 DN - - - + -
MEK1 Act - - - - +
Blot
α-MyoD
α-HA-MEK1
α-Myogenin
α–HEB
1 2 3 4 5

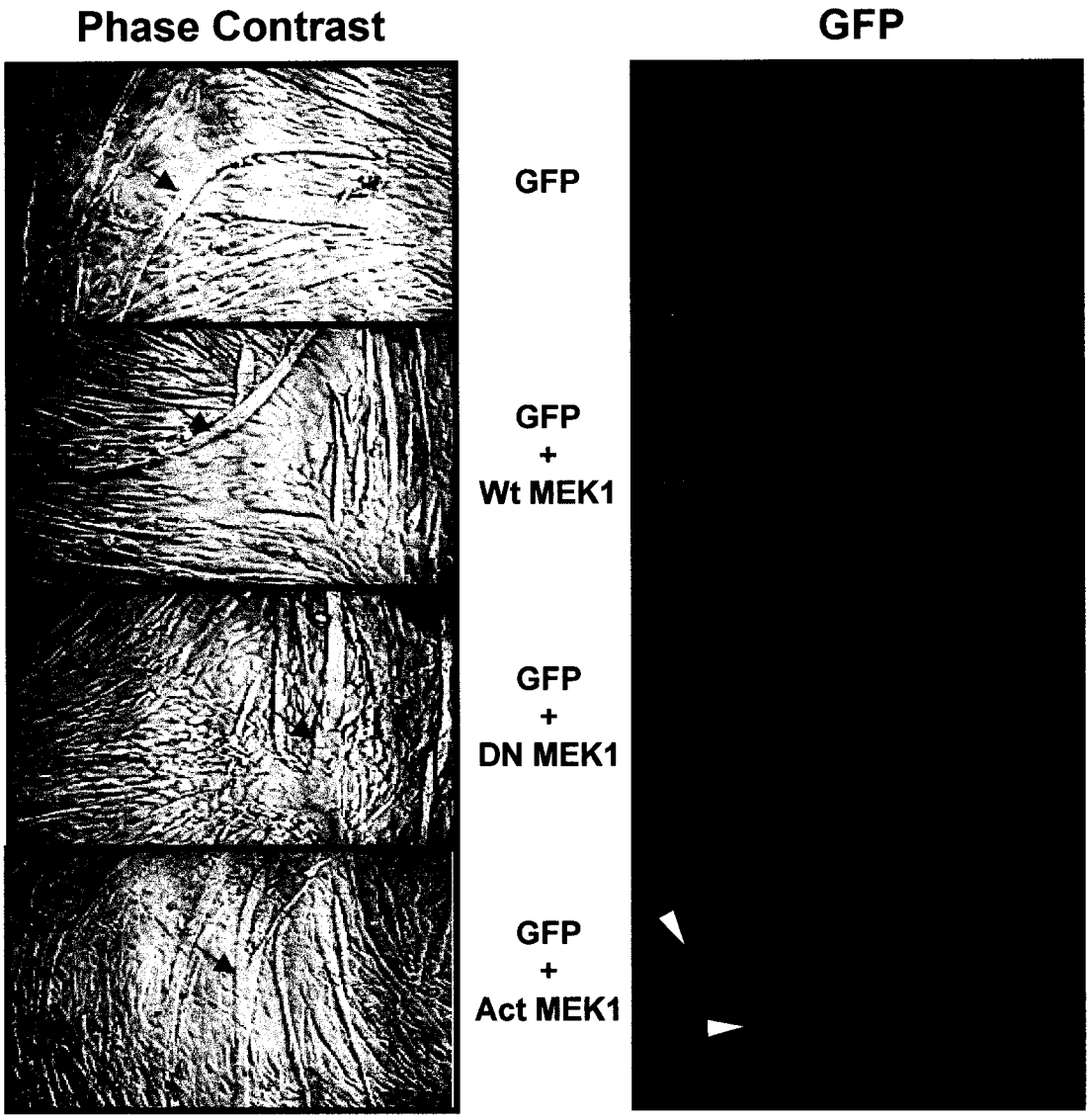
form of MEK1 yielded single, non-differentiated GFP-positive cells (Figure 4.4; bottom right panel, white arrowheads). The lack of GFP-positive multinucleated myotubes suggests activated MEK1 inhibits myogenesis. This defect appears to be cell autonomous as myotubes are detected in neighboring, GFP-negative cells (Figure 4.4; bottom panels, red arrows).

Together, the inability of the myogenic factors to transactivate myogenic reporter vectors and convert fibroblasts to the myogenic lineage when coexpressed with the activated form of MEK1 suggests an important role for the MAPK pathway in regulating myogenesis. Moreover, activated MEK1 represses terminal differentiation of established C2C12 myoblasts, further supporting a negative role for MAPK signaling during myogenesis. Importantly, the level of regulation is not at the level of protein stability as steady-state levels of MyoD are unchanged irrespective of the MEK1 mutant coexpressed in 10T1/2 fibroblasts.

MyoD as a kinase substrate of MAPKs

The main function of the MAPK pathway is to alter transcription factor activity by phosphorylation. The next set of experiments examined whether MyoD represents a direct downstream target of MAPK phosphorylation. Within the amino- (N-) and carboxyl- (C-) terminal domains of MyoD are several putative proline-directed serine/threonine residues (see Figure 1.2). To assess whether MyoD represents a direct downstream target of the ERK or JNK pathways, *in vitro* kinase assays were performed. To activate these kinases, serum starved 10T1/2 cells were stimulated with TPA (200 ng/ml) for ERK activation (Figure 4.5A) or UV-irradiation (40 J/m^2) for JNK activation (Figure 4.5B). The kinetics of activation was assessed by immunoprecipitation (IP)-

Figure 4.4: Activated MEK1 interferes with C2C12 myoblast differentiation. C2C12 myoblasts were transfected with the mutant versions of MEK1 and a constitutively expressed green fluorescent protein (GFP) reporter vector. Terminal differentiation was assessed by the formation of morphologically distinct multinucleated myotubes (phase contrast; red arrows). Transfected myoblasts undergo normal differentiation when wild-type and dominant negative forms of MEK1 are coexpressed. However, when the activated form of MEK1 is expressed in C2C12 myoblasts, the cells are unable to form multinucleated myotubes (white arrowheads). The effect appears to be cell autonomous as there is no differentiation defect found in surrounding, non-GFP expressing cells (red arrows in GFP and activated MEK1 phase and fluorescence photomicrographs). Essentially all cells transfected in this manner are positive for the HA-tagged MEK1 (see Figure 4.10). Left panels were taken using phase contrast optics and all photomicrographs were taken using a 10X objective.


Phase Contrast
GFP
GFP
GFP + Wt MEK1
GFP + DN MEK1
GFP + Act MEK1
5 Days Differentiation

kinase assays and kinase-dependent transfer of radiolabelled phosphate group to known ERK and JNK control kinase substrates. It can be seen that TPA stimulation leads to maximal ERK activation by 5 minutes as measured by phosphorylation of GST-ELK1 (Figure 4.5A). By contrast, maximal JNK activation was achieved 30 minutes after UV-irradiation as measured by GST-jun phophorylation (Figure 4.5B). The time points of maximal kinase activation were used to determine if MyoD represents a substrate of ERK and/or JNK kinases.

In a similar fashion as described above, serum-starved 10T1/2 fibroblasts were stimulated with TPA or UV-irradiation and IP-kinase assays were performed using GST-MyoD and GST-E12 fusion proteins as substrates (Figure 4.6). It can be seen that while activated ERKs efficiently phosphorylate the control GST-ELK1 substrate (Figure 4.6A; lanes 3 & 4), full-length MyoD, or E12 were not targeted for phosphorylation (Figure 4.6A; lanes 5 & 6, and 7 & 8, respectively). Similarly, MyoD/E12 heterodimers were not phosphorylated by ERK kinases (Figure 4.6A; lanes 13 & 14). To determine if conformational changes due to DNA binding yield available targets, an oligonucleotide representing an E-box was included. Inclusion of the E-box oligonucleotide did not result in MyoD homodimers (Figure 4.6A; lanes 9 & 10), E12 homodimers (Figure 4.6A; lanes 11 & 12) or MyoD/E12 heterodimers (Figure 4.6A; lanes 15 & 16) being phosphorylated by ERKs. It should be noted that under the conditions used for these experiments, MyoD and E12 form homo- and heterodimers that bind the synthetic E-box oligonucleotide as determined by electrophoretic mobility shift assay (data not shown). Similar experiments were performed using activated JNK and it was determined that MyoD and E12 homo- and heterodimers are not substrates for JNK phosphorylation

whether in the presence or absence of an E-box oligonucleotide (Figure 4.6B). This result is supported by data showing that coexpression of the MRFs with mutant forms of the JNK upstream activator, SEK1, has no effect on muscle-specific gene expression (data not shown).

To further examine the potential of intramolecular folding hiding putative phosphoacceptor sites, GST fusions of the N-terminal (amino acids 1-95) and C-terminal (amino acids 174-318) portions of MyoD were examined as potential kinase substrates (Figure 4.7). As observed with full-length MyoD, neither the N- or C-terminal regions represent substrates for ERK (Figure 4.7A) or JNK (Figure 4.7B) kinases. This data clearly demonstrates that MyoD does not represent a kinase substrate of the ERK or JNK pathways and suggests another mechanism is involved in MEK1-mediated repression of myogenesis.

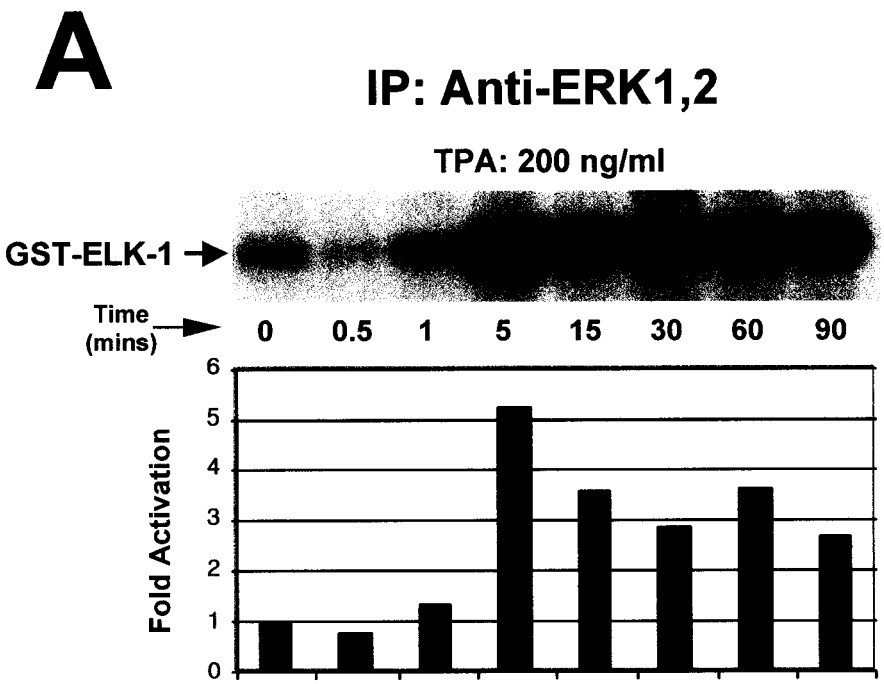
Subcellular Localization of MyoD and MEK1

Changes in the subcellular localization of several molecules is an important level of regulation. MyoD is considered a constitutively nuclear phosphoprotein (Tapscott *et al.*, 1988) with localization being mediated by two putative nuclear localization signals within the basic-helix 1 region of the molecule (Vandromme *et al.*, 1995). Activation of the MAPKs leads to their nuclear translocation, allowing for direct phosphorylation of transcription factors (Chen *et al.*, 2001). There is increasing evidence that while MEK1 contains a strong nuclear export signal within the N-terminal region of the molecule (Tolwinski *et al.*, 1999), growth factor stimulation, inhibition of the nuclear export machinery and cell cycle dependent regulatory mechanisms lead to the localization of activated MEK to the nucleus (Fukuda *et al.*, 1997a; Fukuda *et al.*, 1997b; Jaaro *et al.*,

Figure 4.5: Kinase activation in serum starved 10T1/2 fibroblasts.

(A) Serum-starved 10T1/2 fibroblasts were stimulated with 200 ng/ml TPA and cell extracts were collected at the indicated times. Immunoprecipitation kinase assays were performed using GST-ELK-1 as a control ERK1/2 substrate. Kinase activity was measured by the transfer of radiolabelled phosphate to the substrate. Quantitation was done using a Molecular Dynamics Phosphorimager. It can be seen that over the time examined ERK1/2 activity peaks after five minutes post-stimulation with approximately 5-fold activation.

(B) Serum-starved 10T1/2 fibroblasts were stimulated with 40 Joules/m^2 (flow rate of 1.5 Joules) and cell extracts were collected at the indicated times. Immunoprecipitation kinase assays were performed using the JNK-specific control substrate GST-jun. Kinase activity was measured by the transfer of radiolabelled phosphate to the substrate. Quantitation was done using a Molecular Dynamics Phosphorimager. It can be seen that maximal JNK activity is achieved 30 minutes post-stimulation and is nearly 16-fold above that of serum-starved cells.


A
IP: Anti-ERK1,2
TPA: 200 ng/ml
GST-ELK-1
Time (mins)
0 0.5 1 5 15 30 60 90
Fold Activation
0 1 2 3 4 5 6

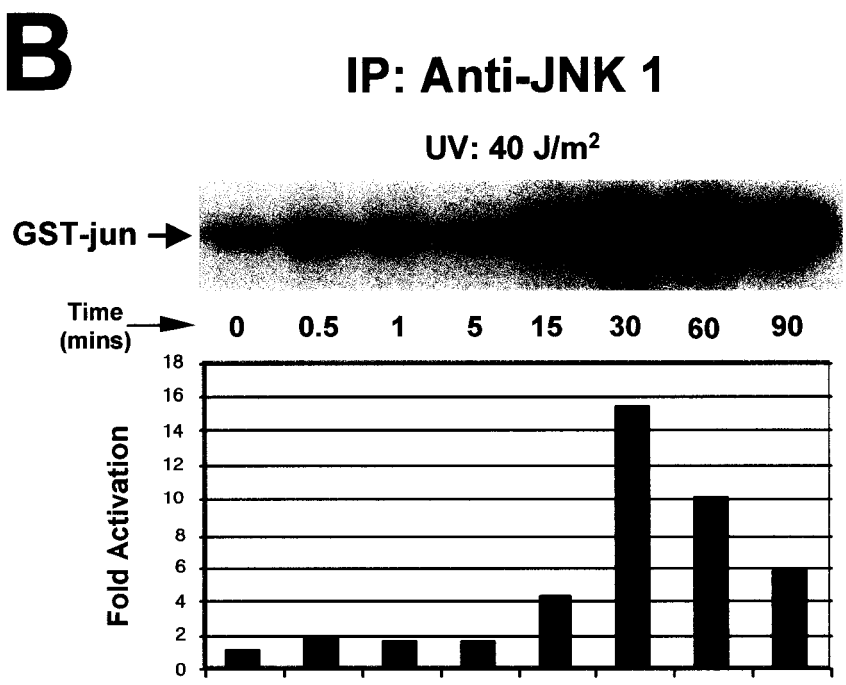
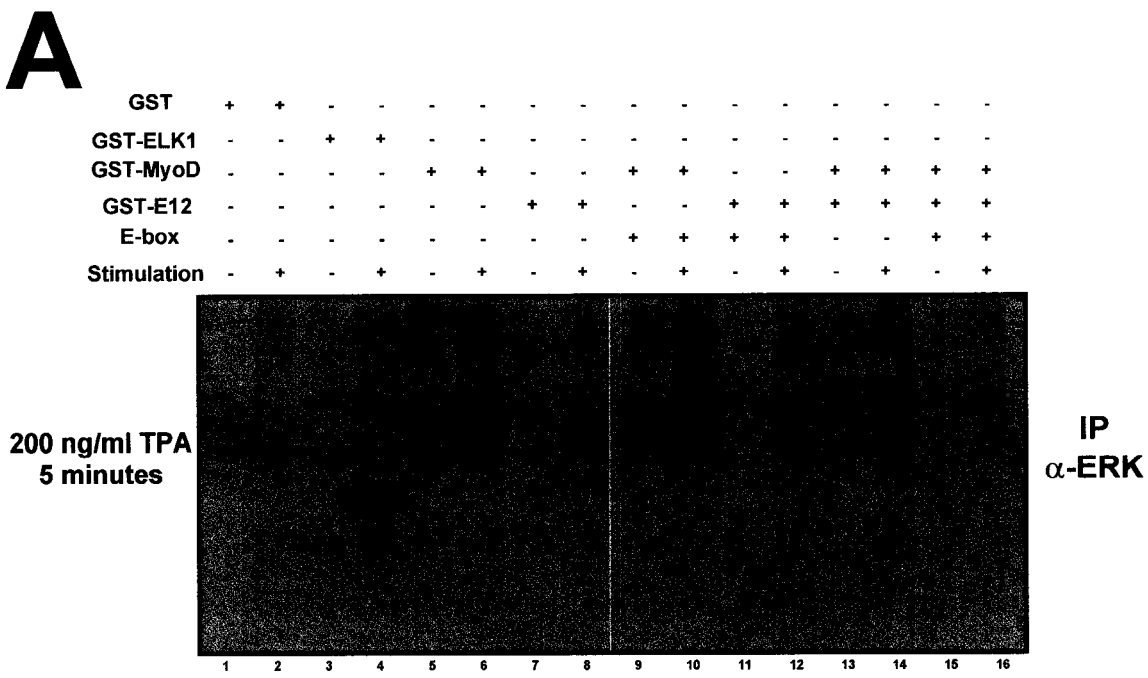

B
IP: Anti-JNK 1
UV: 40 J/m2
GST-jun
Time (mins)
0 0.5 1 5 15 30 60 90
Fold Activation
0 2 4 6 8 10 12 14 16 18

Figure 4.6: MyoD, E12 or heterodimers are not ERK or JNK substrates.

(A) Immunoprecipitation kinase assays were performed using TPA-stimulated serum-starved 10T1/2 extracts and substrates were assessed for ERK1/2 phosphorylation. While the control GST-ELK1 substrate is efficiently phosphorylated by ERKs after TPA stimulation (compare lanes 3 and 4), it can be seen that full-length MyoD (lanes 5 and 6), full-length E12 (lanes 6 and 7) or, MyoD/E12 heterodimers (lanes 13 and 14) are not ERK1/2 kinase substrates. Moreover, in the presence of an oligonucleotide representing a cognate E-box, neither protein alone (MyoD lanes 9 and 10; E12 lanes 11 and 12) or heterodimers (lanes 15 and 16) represent ERK1/2 substrates.

(B) Immunoprecipitation kinase assays were performed using UV-irradiated serum-starved 10T1/2 extracts and substrates were assessed for whether they are specific JNK targets. While the control GST-jun substrate is efficiently phosphorylated by JNK after UV-irradiation (compare lanes 3 and 4), full-length MyoD (lanes 5 and 6), full-length E12 (lanes 6 and 7) and MyoD/E12 heterodimers (lanes 13 and 14) are not targeted by JNKs. Moreover, in the presence of an oligonucleotide representing a cognate E-box, neither protein alone (MyoD lanes 9 and 10; E12 lanes 11 and 12) or, as a heterodimer (lanes 15 and 16), represent JNK substrates.


A
GST + + - - - - - - - - - - - - - -
GST-ELK1 - - + + - - - - - - - - - - - -
GST-MyoD - - - - + + - - + + - - + + + +
GST-E12 - - - - - - + + - - + + + + + +
E-box - - - - - - - - + + + + - - + +
Stimulation - + - + - + - + - + - + - + - +
200 ng/ml TPA
5 minutes
IP
α-ERK
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

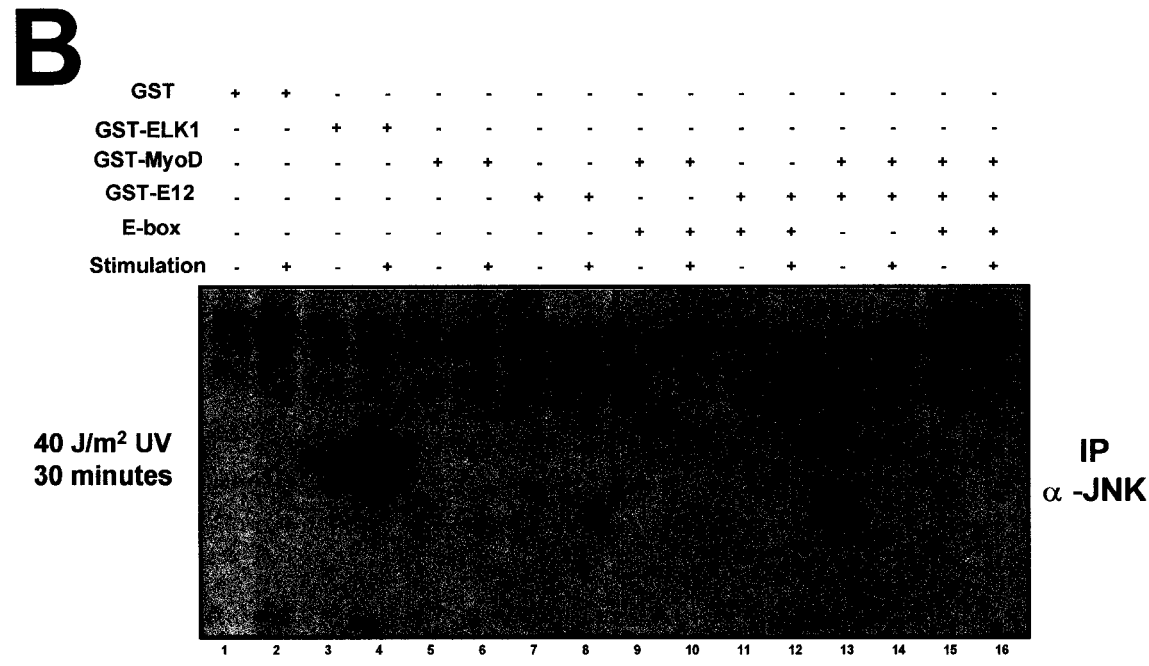
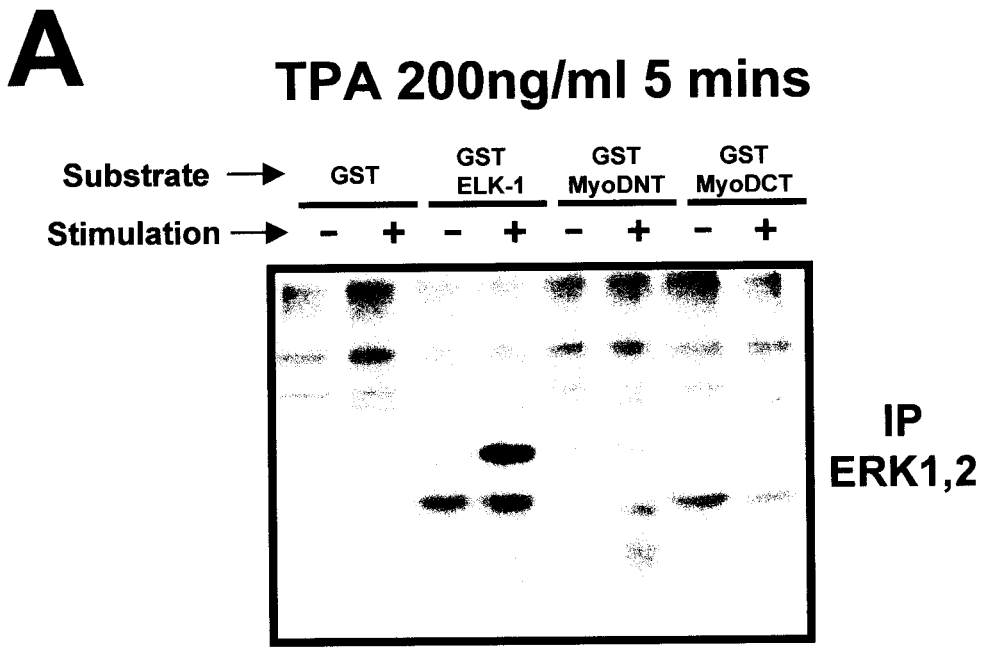
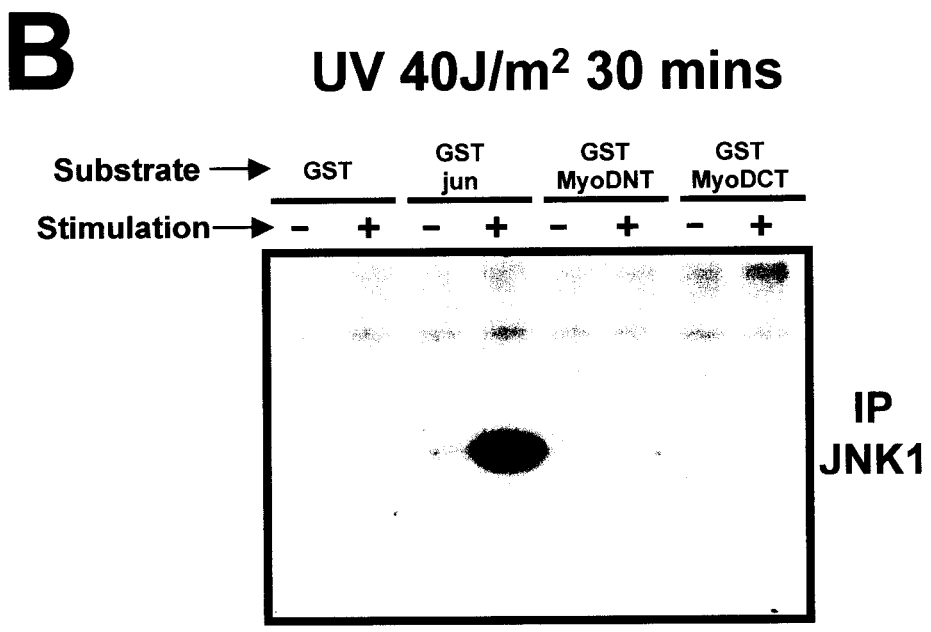

B
GST + + - - - - - - - - - - - - - -
GST-ELK1 - - + + - - - - - - - - - - - -
GST-MyoD - - - - + + - - + + - - + + + +
GST-E12 - - - - - - + + - - + + + + + +
E-box - - - - - - - - + + + + - - + +
Stimulation - + - + - + - + - + - + - + - +
40 J/m2 UV
30 minutes
IP
α -JNK
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4.7: N-terminal and C-terminal portions of MyoD are not kinase substrates. To ensure that secondary structure of full-length MyoD protein did not conceal putative phophoacceptor sites, amino-terminal (amino acids 1-95) and carboxyl-terminal (amino acids 174-318) portions of MyoD, fused to GST, were assayed for their ability to be phosphorylated by ERKs (A) or JNKs (B). It can be seen that in both cases control substrates were readily phosphorylated by ERKs (GST-Elk1) and JNKs (GST-jun) but neither the amino- or carboxyl-terminal portions of MyoD represent targets by either kinase pathway.


A
TPA 200ng/ml 5 mins
Substrate
GST
GST ELK-1
GST MyoDNT
GST MyoDCT
Stimulation
- + - + - + - +
IP ERK1,2


B
UV 40J/m2 30 mins
Substrate
GST
GST jun
GST MyoDNT
GST MyoDCT
Stimulation
- + - + - + - +
IP JNK1

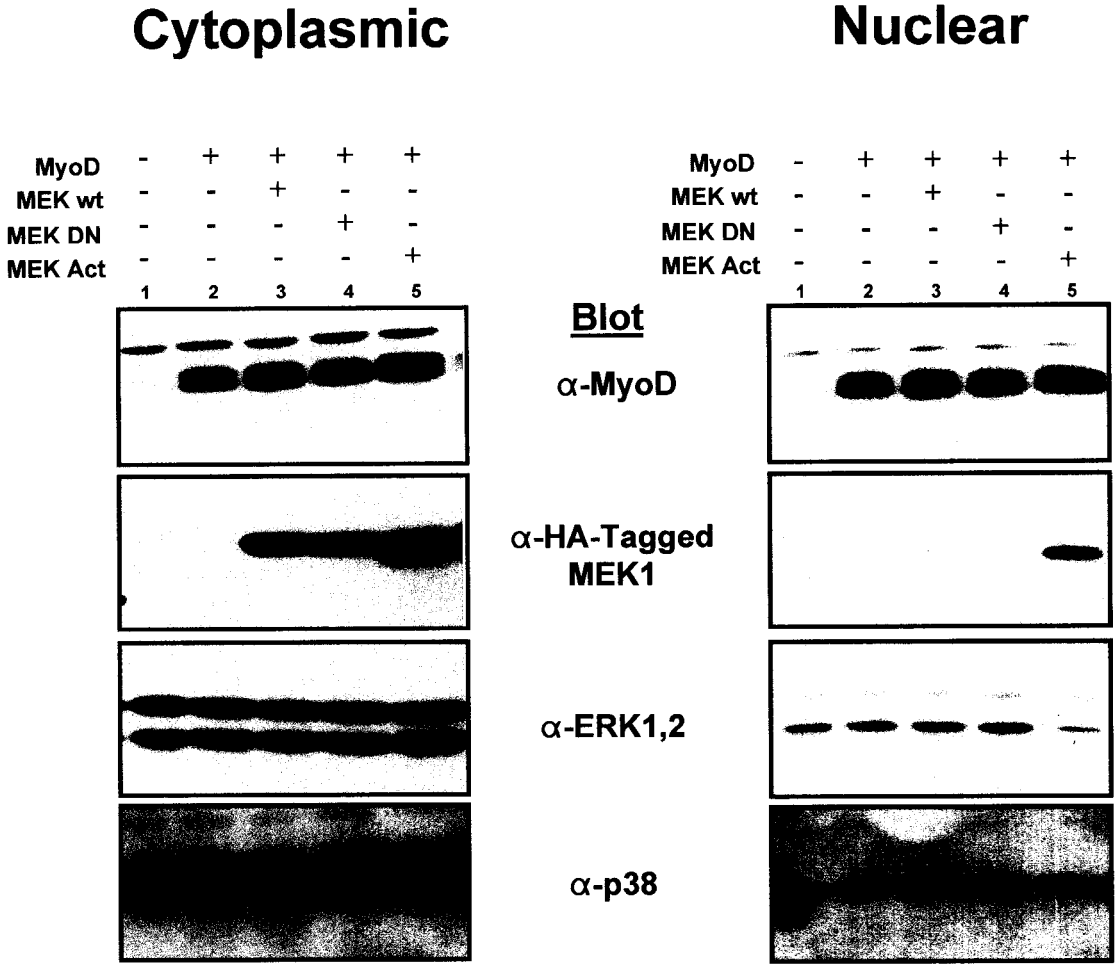
1997; Tolwinski *et al.*, 1999). Importantly, MEK export from the nucleus may represent a mechanism of relocalizing the ERKs to the cytoplasm, thereby halting growth factor-induced changes in gene expression (Rubinfeld *et al.*, 1999). To determine if coexpression of activated MEK1 affects nuclear localization of MyoD, the subcellular distribution of MyoD and mutant MEKs was analyzed in transfected 10T1/2 fibroblasts.

Initial experiments examined protein localization of transfected proteins by using a sequential extraction protocol that permits the isolation of proteins from the cytoplasmic and nuclear compartments of the cell. Subcellular localization patterns were determined by SDS-PAGE and immunoblotting (Figure 4.8). It can be seen that the expression level of MyoD in the nuclear fraction is unaffected by coexpression of activated MEK1 (Figure 4.8; lanes 2-5, nuclear top panel). It should be noted that detection of MyoD in the cytoplasmic fraction is likely due to high levels of exogenous expression as C2C12 myoblasts extracted in the same manner show essentially all MyoD within the nuclear compartment (data not shown). Immunodetection of the HA-tagged MEK1 mutants shows that wild-type and dominant negative forms of MEK1 are found only in the cytoplasmic fraction (Figure 4.8; compare nuclear and cytoplasmic, lanes 3 & 4). By contrast, activated MEK1 is in both cytoplasmic and nuclear fractions (Figure 4.8; compare lane 5 in left and right panels). Levels of ERK 1/2 are moderately reduced within the nuclear fraction in cells expressing activated MEK1 whereas p38 nuclear levels remain unchanged (Figure 4.8).

To confirm the presence of activated MEK1 in the nucleus, 10T1/2 fibroblasts transfected with MyoD, mutant MEKs and a constitutively expressed GFP vector were examined by immunofluroescence (Figure 4.9). As seen with cellular fractionation,

Figure 4.8: MyoD nuclear localization is unaffected by activated MEK1.

Transfected 10T1/2 fibroblasts were fractionated into cytoplasmic and nuclear extracts. Immunoblotting was performed using 30 μg of cytoplasmic protein and 10 μg of nuclear protein and blots were probed as indicated. Nuclear MyoD levels remain unchanged suggesting that activated MEK1 does not affect subcellular localization of MyoD. Interestingly, the nuclear fraction demonstrates the presence of the HA-tagged activated MEK1. Levels of ERK1/2 within the nuclear fraction appear reduced whereas the p38 MAPK levels are unchanged.


Cytoplasmic
Nuclear
MyoD - + + + +
MEK wt - - + - -
MEK DN - - - + -
MEK Act - - - - +
1 2 3 4 5
Blot
α-MyoD
α-HA-Tagged MEK1
α-ERK1,2
α-p38

MyoD is detected in nuclei of GFP positive cells when coexpressed with all mutant forms of MEK1 (Figure 4.9; yellow arrows). Immunolocalization of wild-type and dominant negative forms of MEK1 show these molecules are only detected in the cytoplasm of GFP-positive cells (Figure 4.9 B & C; white arrows). By contrast, immunodetection of activated MEK1 clearly shows that MEK1 is detected in both the cytoplasm and nucleus of transfected, GFP positive cells (Figure 4.9 D; white arrow). This confirms data from other laboratories showing that this mutant of activated MEK1 readily localizes to the nucleus due to the deletion of an N-terminal nuclear export signal (Tolwinski *et al.*, 1999). Moreover, nuclear localization of MEK1 likely represents a vital aspect of MRF-mediated gene expression as previous studies showing no effect of activated MEK1 on MRF-mediated gene expression used a full-length mutant containing the nuclear export signal (Ramrocki *et al.*, 1997).

An absolute requirement for MEK1 to be localized within the nucleus is that it must be serine phosphorylated and in an activated state (Tolwinski *et al.*, 1999). Clearly, the form of MEK1 used for these studies resides within the nuclear compartment suggesting this may be important for regulating MRF activity. To assess whether myoblasts demonstrate nuclear localization of endogenous MEK1 upon MAPK activation, proliferating and differentiating C2C12 cells were stimulated with TPA and MEK localization was determined by immunofluorescence (Figure 4.10). It can be seen that stimulation of proliferating C2C12 myoblasts leads to an increase in the number of cells with MEK1 localized within the nucleus (Figure 4.10A). Interestingly, C2C12 cells cultured for 48 hours under differentiating conditions show no increase in MEK1 nuclear localization after TPA stimulation (Figure 4.10B). While the data shows C2C12

Figure 4.9: Immunofluorescence reveals the presence of activated MEK1 in the nucleus.

To confirm the cell fractionation data, 10T1/2 fibroblasts were transfected with MyoD alone (A), MyoD and wild-type MEK1 (B), MyoD and dominant negative MEK1 (C) or MyoD and activated MEK1 (D). Transfected cells were marked by the inclusion of a constitutively expressed green fluorescent protein (GFP) vector. Cells were fixed and processed for immunofluorescence after 48 hours under differentiation conditions. Immune complexes of MyoD and HA were detected using a secondary anti-mouse rhodamine-conjugated antibody. Cells were stained with Hoechst dye to show all nuclei. It can be seen that in all cases, cells that are GFP positive show the presence of MyoD in the nucleus (yellow arrows). In the case of HA-immunofluorescence, there is none detected in the control transfected cells whereas both wild-type and dominant-negative forms are detected in the cytoplasmic compartment as indicated by the lack of nuclear staining (white arrows). By contrast, cells transfected with the activated form of MEK1 demonstrate staining in both cytoplasmic and nuclear compartments (D; white arrow) confirming the biochemical data that this form of activated MEK1 can reside within the nuclear compartment of cells even under low-mitogen conditions. Photomicrographs were obtained using a 20X objective.

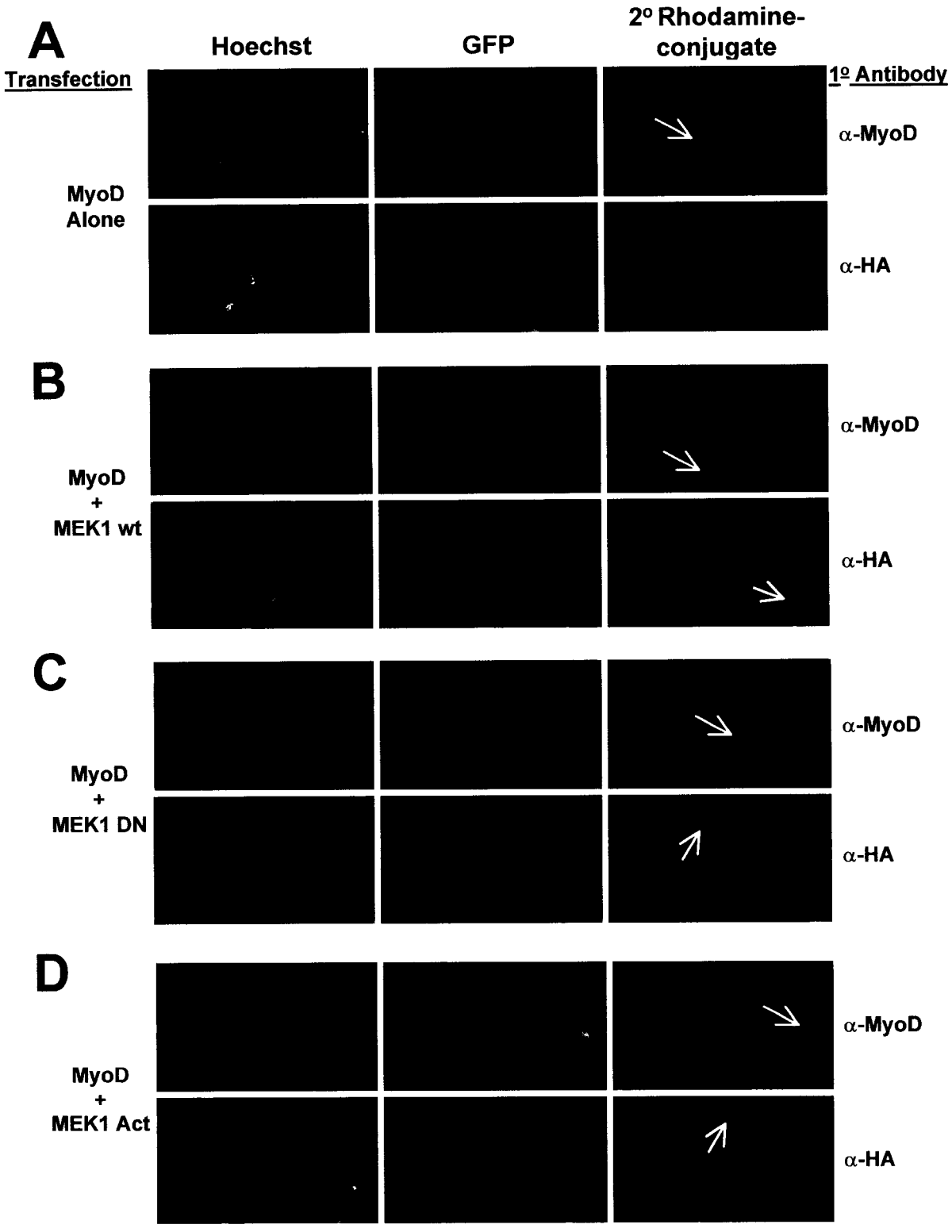
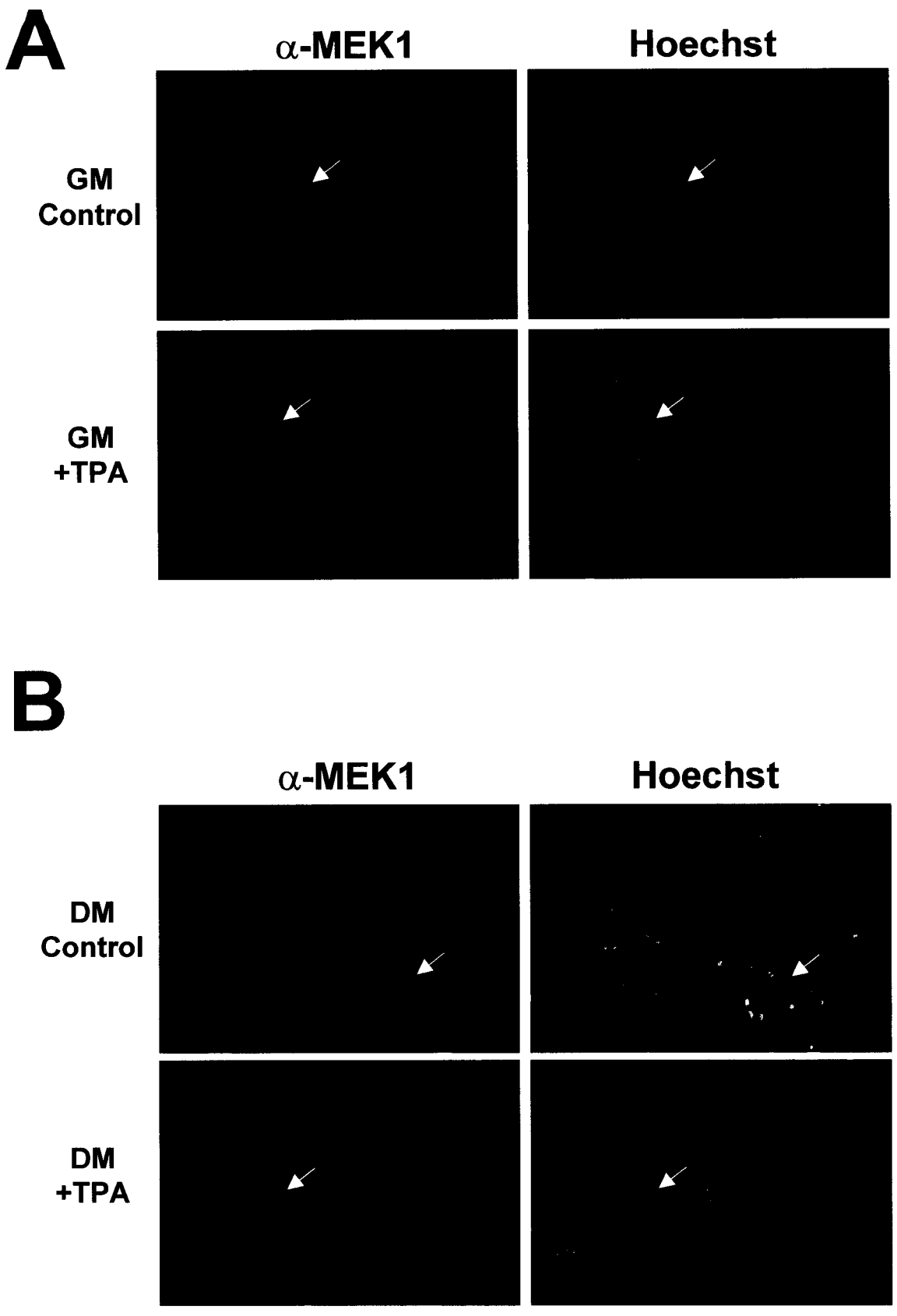

A
Hoechst
GFP
2° Rhodamine-conjugate
Transfection
1º Antibody
MyoD Alone
α-MyoD
α-HA
B
MyoD + MEK1 wt
α-MyoD
α-HA
C
MyoD + MEK1 DN
α-MyoD
α-HA
D
MyoD + MEK1 Act
α-MyoD
α-HA

Figure 4.10: TPA-stimulation leads to MEK1 presence in the nucleus of myoblasts. To address the potential that MEK1 can be found in the nucleus upon MAPK pathway stimulation, proliferating C2C12 myoblasts (A) and differentiating myotubes (B) were stimulated for 30 minutes with DMSO (control) or TPA (200 ng/ml). Cells were fixed 30 minutes post-stimulation and processed for MEK1 immunofluorescence. Immunofluorescent localization of MEK1 reveals translocation to the nucleus in dividing myoblasts stimulated with TPA (left panels in A). By contrast, stimulation of differentiating C2C12 cells cultured for 48 hours under differentiating conditions does not result in the nuclear localization of MEK1 after TPA stimulation (left panels in B). All nuclei are stained with Hoechst dye, white arrows indicate the same cells and photomicrographs were obtained using a 20X objective.

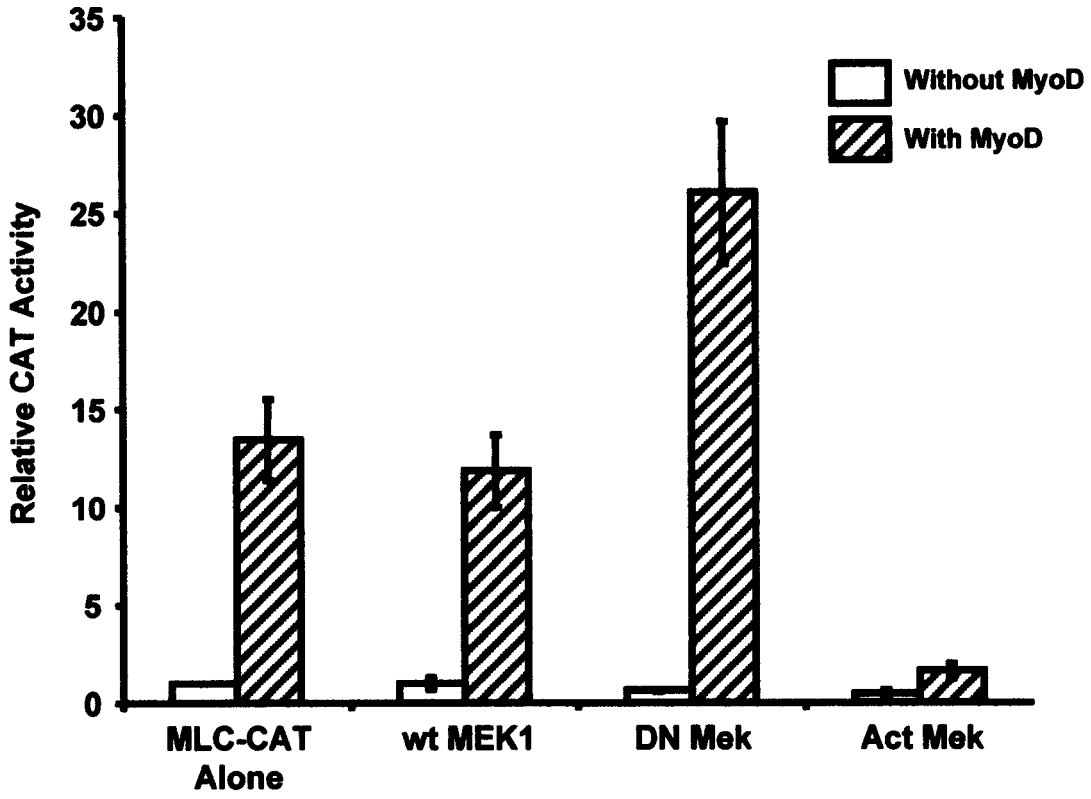

A
α-MEK1
Hoechst
GM
Control
GM
+TPA
B
α-MEK1
Hoechst
DM
Control
DM
+TPA

myoblasts respond to growth factor stimulation in a similar manner as observed in other cell types, the difference between proliferating and differentiating cells suggests that the MAPK signaling module is differentially regulated in the proliferating versus differentiating phases of the myogenic program.

MEK1 inhibition advances differentiation and affects myotube formation

The data presented thus far demonstrates that MAPK signaling negatively regulates myogenesis in a fashion similar to that observed with peptide growth factor stimulation. The next set of experiments sought to determine if inhibition of MAPK signaling serves to stimulate myogenic differentiation. Data presented earlier clearly showed that activated and dominant negative forms of MEK1 differentially modulate MyoD-mediated gene expression under differentiating conditions (Figure 4.11B). To specifically address MAPK inhibition, a similar experiment was carried out with the exception that transfected cells were harvested after 48 hours in growth promoting (10% FCS) conditions (Figure 4.11). In the presence of high serum, MyoD does not activate the MLC-CAT reporter vector as well as seen under low-mitogen conditions. However, as observed under differentiating conditions, coexpression of activated MEK1 greatly reduces MyoD transcriptional activity. By contrast, MyoD-mediated gene expression is increased when dominant negative MEK1 is coexpressed (Figure 4.11; DN MEK1, with MyoD). While this indicates that MAPK inhibition positively influences MyoD-mediated reporter gene activation, MF20 immunocytochemistry demonstrated no increase in terminal differentiation (data not shown), suggesting alternate mechanisms involved with negatively regulating myogenic differentiation.

Figure 4.11: Dominant negative MEK1 increases MyoD-mediated gene expression. To assess whether inhibition of MAPK signaling enhances MyoD-mediated gene expression, C3H10T1/2 fibroblasts were cotransfected with plasmids encoding MyoD, mutant MEKs and the MLC-CAT reporter. Cells were harvested after 48 hours under growth conditions (10% FCS) and CAT assays were performed. As with previous data, inclusion of the activated form of MEK1 dramatically reduced the level of MyoD-mediated transcriptional activity. By contrast, dominant negative MEK1 increased MyoD-mediated gene expression in a similar manner as that observed under differentiation conditions. This suggests inhibition of MAPK signaling enhances MyoD-mediated gene activation under conditions of high mitogenic signaling. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=9).


35
30
25
20
15
10
5
0
Relative CAT Activity
Without MyoD
With MyoD
MLC-CAT Alone
wt MEK1
DN Mek
Act Mek

The next set of experiments took advantage of a synthetic MAPK signaling pathway inhibitor. This inhibitor, U0126, permits Raf-mediated activation of MEK1/2 but blocks activated MEK nuclear translocation and MEK-dependent ERK activation (Favata *et al.*, 1998). To demonstrate the effectiveness of U0126, serum-starved 10T1/2 fibroblasts were stimulated with TPA in the absence or presence of U0126 and activation of MEK and ERK kinases was assessed by immunoblotting using antibodies specific for the activated, phosphorylated forms of these molecules (Figure 4.12A). Unstimulated cells show essentially no MEK activation and only a very low level of ERK phosphorylation (Figure 4.12A; lane 1). Stimulation of cells with TPA leads to a large increase in the amounts of both activated MEK and activated ERK detected (Figure 4.12A; lane 2). By contrast, inclusion of U0126 results in the efficient activation of MEK1 whereas the downstream phosphorylation of ERKs is similar to that of unstimulated cells (Figure 4.12A; compare lanes 1 & 3, p-ERK blot). Under all conditions examined, similar levels of MEK1/2 and ERK1/2 proteins are detected (Figure 4.12A; bottom two panels).

To illustrate the effectiveness of U0126, 10T1/2 fibroblasts were transfected with MyoD and activated MEK1 in the absence and presence of the chemical inhibitor. After 48 hours in differentiation medium, cells were harvested and the ability of U0126 to restore MyoD function was assessed by examining the levels of myogenin expression (Figure 4.12B). As previously observed (Figure 4.3), the ability of MyoD to activate myogenin is greatly reduced when coexpressed with activated MEK1 (Figure 4.12B; compare lanes 1 and 2). By contrast, cells cultured in the presence of U0126 lead to increased myogenin expression, indicating that U0126 blocks the negative effects of

activated MEK1 under differentiating conditions. (Figure 4.12B; lane 4). Levels of activated MEK1 and HEB are similar while the control sample shows increased MyoD expression (Figure 4.12B; lane 1).

To further understand the role of the MAPK pathway in regulating myogenesis, the effects of U0126 were examined on cultured C2C12 myoblasts. Treatment of C2C12 myoblasts with U0126 under growth conditions does not increase the number of terminally differentiated cells as measured by MF20 immunocytochemistry (data not shown). Inclusion of U0126 in medium of differentiating C2C12 does not affect the expression of markers of terminal differentiation, as assessed by MF20 immunofluorescence (Figure 4.13). However, under differentiating conditions, U0126 appears to facilitate myoblast cell cycle withdrawal as assessed by total nuclei seen by Hoechst staining (Figure 4.13; right panels) and causes a severe fusion defect, as assessed by the lack of multinucleated myotubes (Figure 4.13; compare cellular morphology of cells shown in left, MF20 panels). This fusion defect mimics a similar phenomenon observed when MAPK signaling is inhibited by overexpression of the MKP-1 phosphatase (Bennett and Tonks, 1997). The dramatic reduction in the total number of nuclei observed after continued presence of U0126 suggests a role for MEK signaling during myoblast cell cycle withdrawal (see below for further discussion).

Together, the data presented in this section provides further support for an important role of MAPK signaling for regulating MRF-mediated gene expression, myoblast cell cycle exit and terminal differentiation. Interestingly, repression of MAPK signaling during the proliferative phase of myogenesis is not sufficient to activate terminal differentiation. This suggests that other pathways are able to override a positive

Figure 4.12: Inhibition of MEK1 signaling by U0126.

(A) Serum-starved 10T1/2 fibroblasts were pretreated for 30 minutes with DMSO or U0126, stimulated with 200 ng/ml TPA and cell extracts were collected after 5 minutes. Stimulation of cells in the absence of U0126 lead to the activation of MEK and ERKs as assessed by immunoblotting with phosphospecific antibodies (compare lanes 1 & 2). In the presence of U0126, MEK is activated whereas downstream ERK activation is blocked, indicating the effectiveness of this drug for the inhibition of downstream MEK activity.

(B) C3H10T1/2 fibroblasts were transfected with MyoD or MyoD and activated MEK1. Cells were harvested after 48 hours in differentiation medium containing DMSO or the MEK inhibitor U0126. Immunoblots were produced using 50 μg of protein extracts and probed as indicated. Coexpression of activated MEK1 reduces expression of myogenin in the absence of U0126. By contrast, inclusion of U0126 in the culture medium leads to an increase of myogenin expression suggesting U0126 interferes with HA-tagged activated MEK1-mediated repression of MyoD transcriptional activation and myogenic conversion.

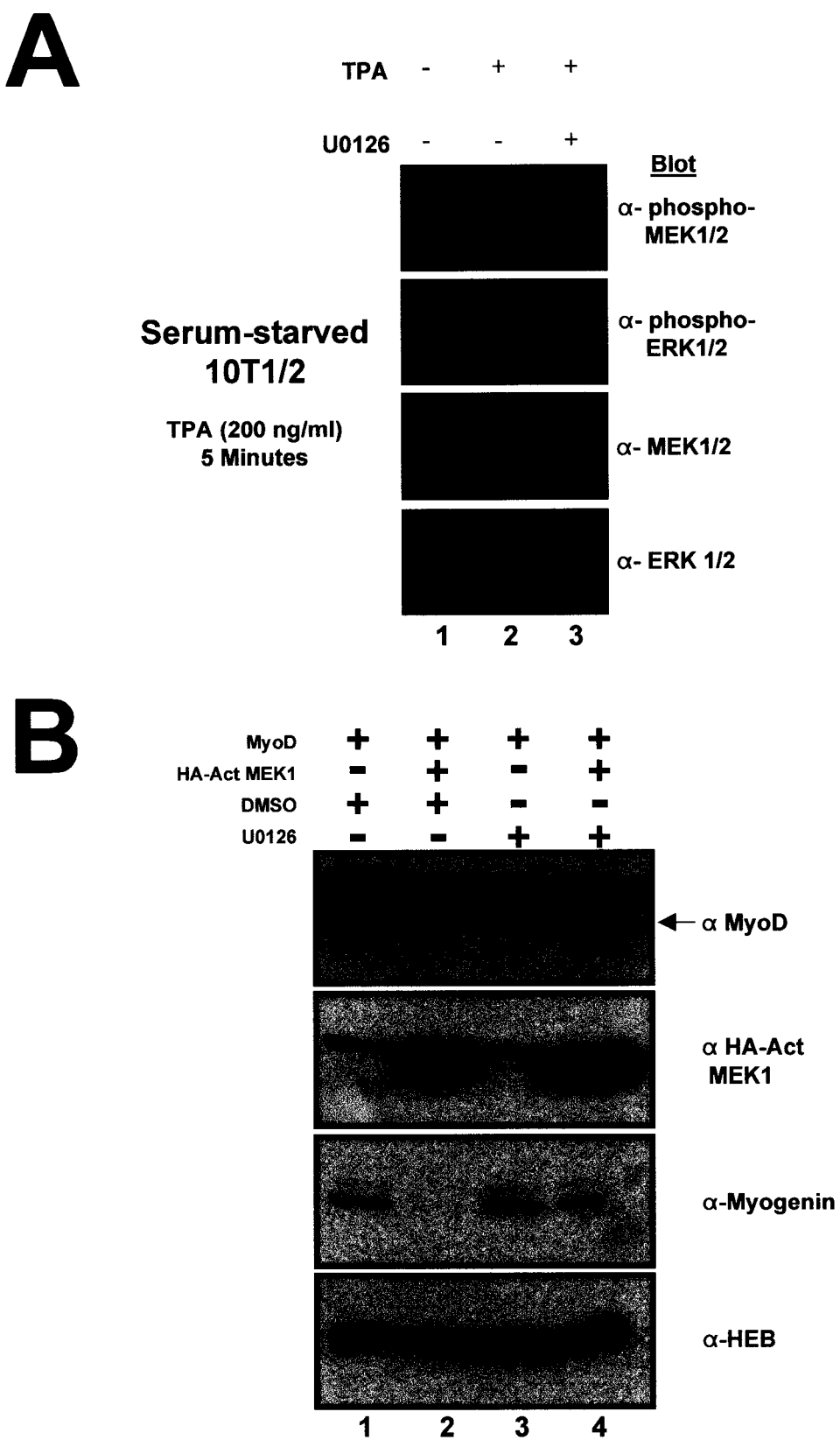
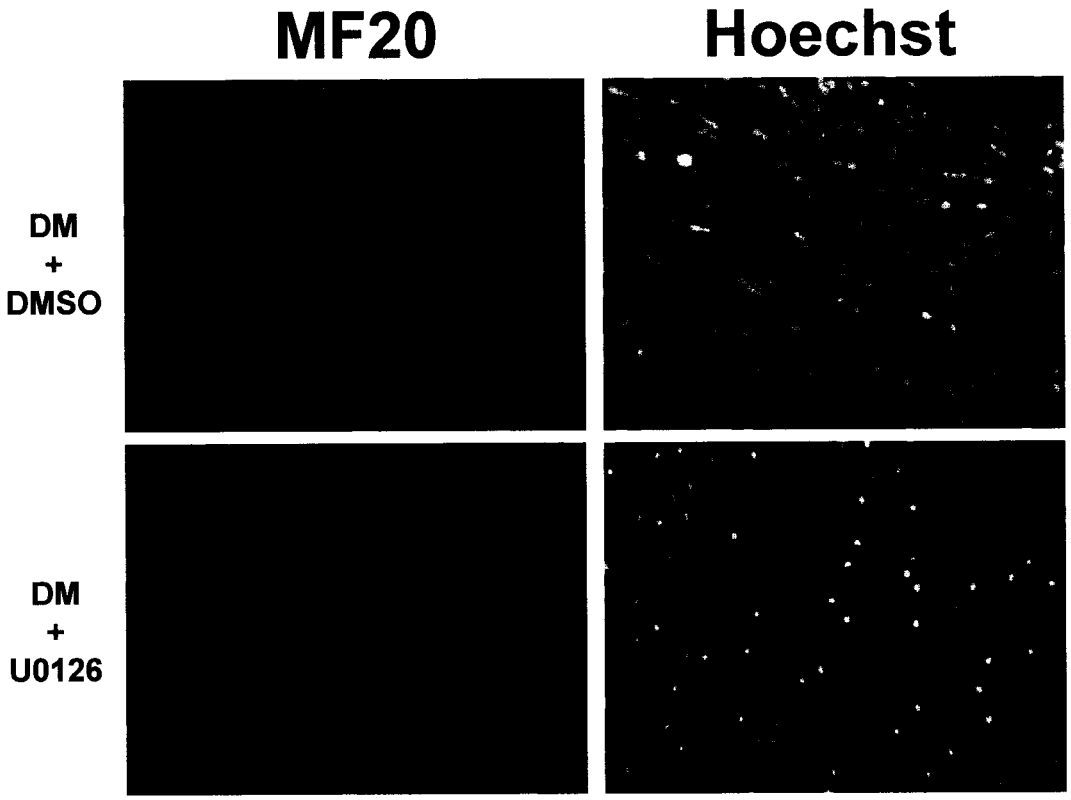

A
TPA - + +
U0126 - - +
Blot
α- phospho-MEK1/2
α- phospho-ERK1/2
α- MEK1/2
α- ERK 1/2
Serum-starved 10T1/2
TPA (200 ng/ml) 5 Minutes
1 2 3
B
MyoD + + + +
HA-Act MEK1 - + - +
DMSO + + - -
U0126 - - + +
α MyoD
α HA-Act MEK1
α-Myogenin
α-HEB
1 2 3 4

Figure 4.13: Inhibition of MAPK signaling affects myotube formation of C2C12 myoblasts but not expression of markers of terminal differentiation.

To understand the functioning of the MAPK pathway on differentiation, C2C12 myoblasts were placed under differentiation conditions in the presence of DMSO or the MEK1 inhibitor U0126 for 5 days. Terminal differentiation was assessed by MF30 immunofluorescence. It can be seen that in the presence of U0126, MF20 is readily detected whereas myotube formation and morphology is severely affected. This suggests an important role of MEK1 activity after the onset of terminal differentiation. All nuclei are marked by Hoechst dye and the reduction in the total number of cells suggests U0126 accelerates terminal differentiation. Photomicrographs were obtained using a 20X objective.


MF20
Hoechst
DM
+
DMSO
DM
+
U0126
5 Days Differentiation

myogenic signal under conditions of MAPK inhibition. However, the MAPK pathway does appear to play an important role as myoblasts exit the cell cycle and terminally differentiate.

MEK1 activation during cell cycle and role during terminal differentiation

An absolute requirement for the myogenic program to proceed is that myoblasts must permanently withdraw from the cell cycle. Of interest is the fact that during the fibroblast cell cycle, MEK becomes activated and localized to the nucleus of cells during the G2-M transition of the cell cycle. The data presented thus far clearly points to a role of MEK1 in regulating MRF-mediated gene expression and terminal differentiation. An important aspect of this regulation appears to be the localization of MEK to the nucleus. Therefore, the next set of experiments were done to examine the nature of how inhibition of MAPK signaling stimulates myoblast cell cycle withdrawal.

Synchronization of *in vitro* cell lines generally involves culturing under low mitogen conditions which induces a G0-state in most cells. Simply refeeding G0-arrested cells with serum-containing medium serves to activate cells to cycle synchronously. Due to the fact that exposure of myoblasts to low-mitogen conditions initiates the myogenic program, a synchronization protocol developed in another laboratory was employed (Kitzmann *et al.*, 1998). This protocol simulates a G0-arrest without activating terminal differentiation or causing cells to lose their myogenic potential. A schematic representation of this protocol is shown (Figure 4.14A). Culturing of cells in methionine/cysteine free medium with low FCS simulates a G0-state for myoblasts and prevents the synthesis of proteins required for differentiation. Release of cells into growth medium and subsequent addition of hydroxyurea causes the accumulation of most

cells at the G1-S boundary (Kitzmann *et al.*, 1998). Use of this protocol established that MyoD and Myf5 protein levels oscillate during distinct phases of the cell cycle, suggesting the decision to differentiate versus proliferate is made by altering the levels of either molecule at very critical cell cycle stages. Importantly, MyoD levels increase during the S- and G2-M phases of the cell cycle whereas Myf5 levels are high during G1, drop during the S- and G2-phases and increase at the G2-M phase to G1 levels (Kitzmann *et al.*, 1998). In light of the fact that MEK1 is activated and translocates to the nucleus at the G2-M phase of the cell cycle, it was hypothesized that this represents a critical time point during the myoblast cell cycle at which time the decision to proliferate or differentiate is made (Figure 4.14B).

To ensure that C2C12 myoblasts were indeed synchronized, time lapse photomicroscopy was done at different time points after the release of cells from the G1-S boundary (Figure 4.15). It can be seen that during the first 7.5 hours post-release, very few dividing cells are observed (Figure 4.15; yellow asterisk marks the same group of cells). By contrast, several cells can be seen transiting through mitosis over the following two hours. The majority of cells undergo mitosis by 10 hours post-release with cells appearing to reenter mitosis approximately 24-hours post-release (Figure 4.14). This series of pictures demonstrates the high level of synchrony of the cultures. Importantly, transfer of cells to differentiation medium for 3 days and MF20 immunocytochemistry shows these cells completely retain their myogenic potential (data not shown).

To examine the status of MEK1 and MyoD, protein extracts were collected at time points post-release and immunoblotting of MyoD, Myf5, MEK, ERKs, HEB and the cyclins were analyzed (Figure 4.16). As previously reported, the levels of MyoD and

Figure 4.14: C2C12 synchronization and MRF oscillations during the cell cycle.

(A) Schematic representation of the protocol employed for synchronizing C2C12 myoblasts (Kitzman *et al*, 1998). Culture of cells in methionine/cysteine, low-serum medium establishes a G0-state without inducing terminal differentiation or causing loss of myogenic identity. Addition of hydroxyurea causes cells to accumulate at the G1-S boundary of the cell cycle. Washing and refeeding cells with fresh growth medium represents time 0 for experiments using this protocol.

(B) Representation of oscillations of MyoD and Myf5 protein levels during the cell cycle and the timing of both MEK activation and nuclear localization. The relative levels do not imply any quantitation but serve only as a visual aid. MEK1 activation and nuclear localization corresponds to the time that MyoD levels are increasing during the G2-M transition of the cell cycle. The hours post-release are the times established in published reports. Adapted from Kitzman *et al* (1998) and Tolwinski *et al* (1999).

A

Seed C2C12
1x10^5/100-mm dish
5x10^4/60-mm dish

24-48 hours

Refeed cells with
DMEM -met/cys
+ 1% dialyzed FCS

36 hours

Refeed cells with
Growth Medium

1 hour

Add hydroxyurea
Final [1mM]

15 hours

Wash 3X with PBS
Refeed with fresh medium
(GM or DM +/- U0126)
Represents G1-S Boundary
and Time=0

B

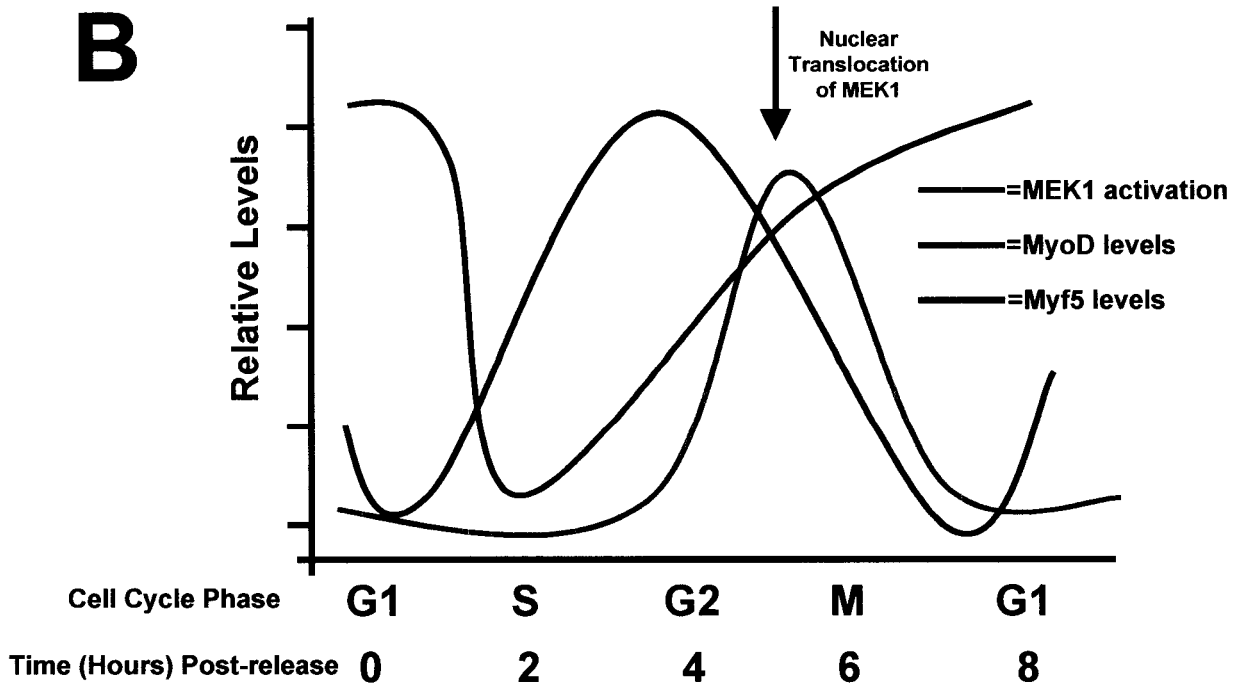
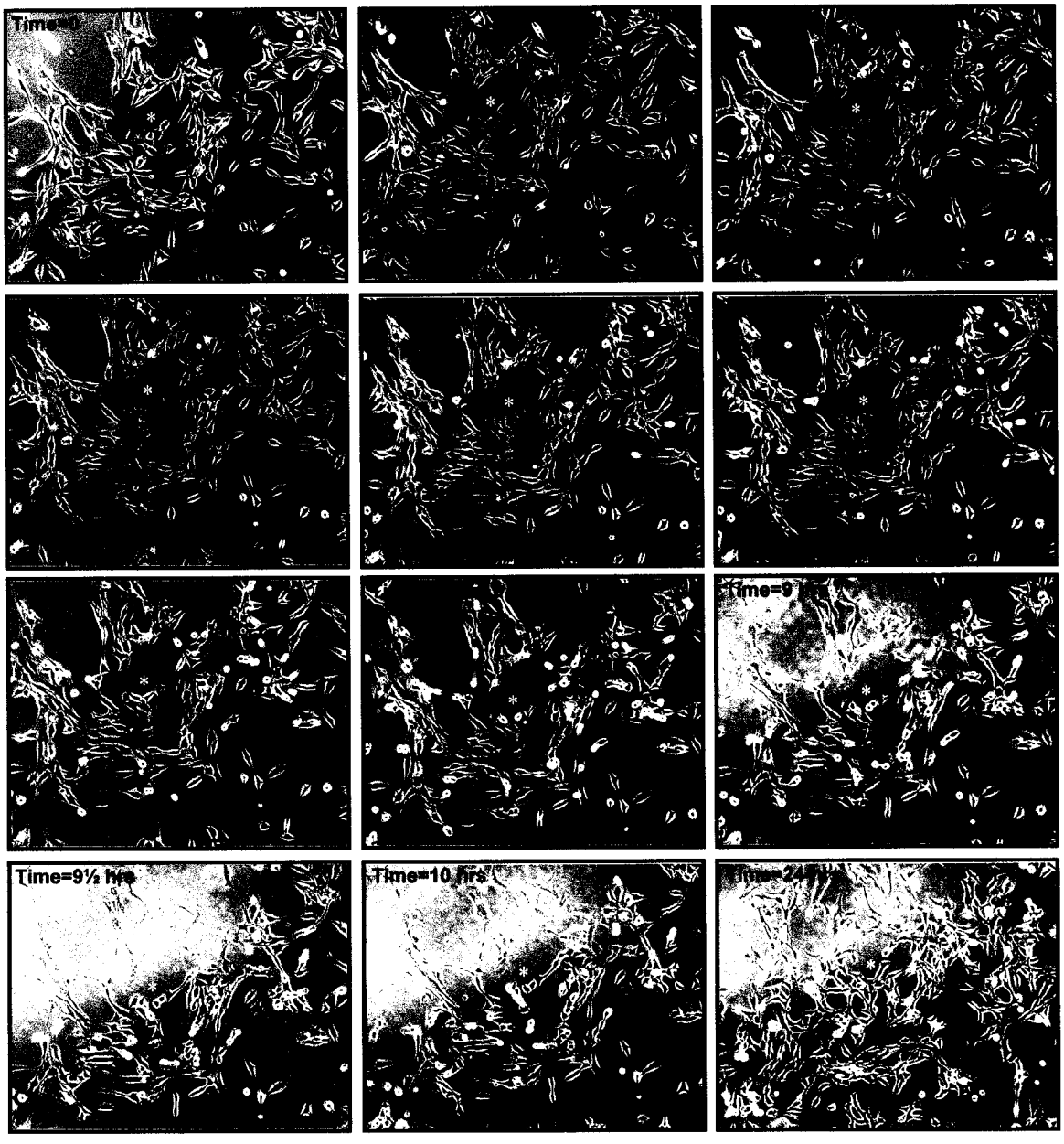


Figure 4.15: Time course photomicroscopy of synchronized C2C12 cells.

C2C12 cells were synchronized to the G1-S boundary and released into growth medium. At the time (in hours) indicated the same region of the cell culture dish was photographed (yellow asterisk marks the same group of cells in all photomicrographs). It can be seen that very few cells undergo mitosis during the first 71/2 hours post-release. During the next 21/2 hours, the majority of cells undergo mitosis synchronously. The process is underway again by the 24-hour period, with cells beginning another round of mitosis. Photomicrographs were obtained using a 10X objective.

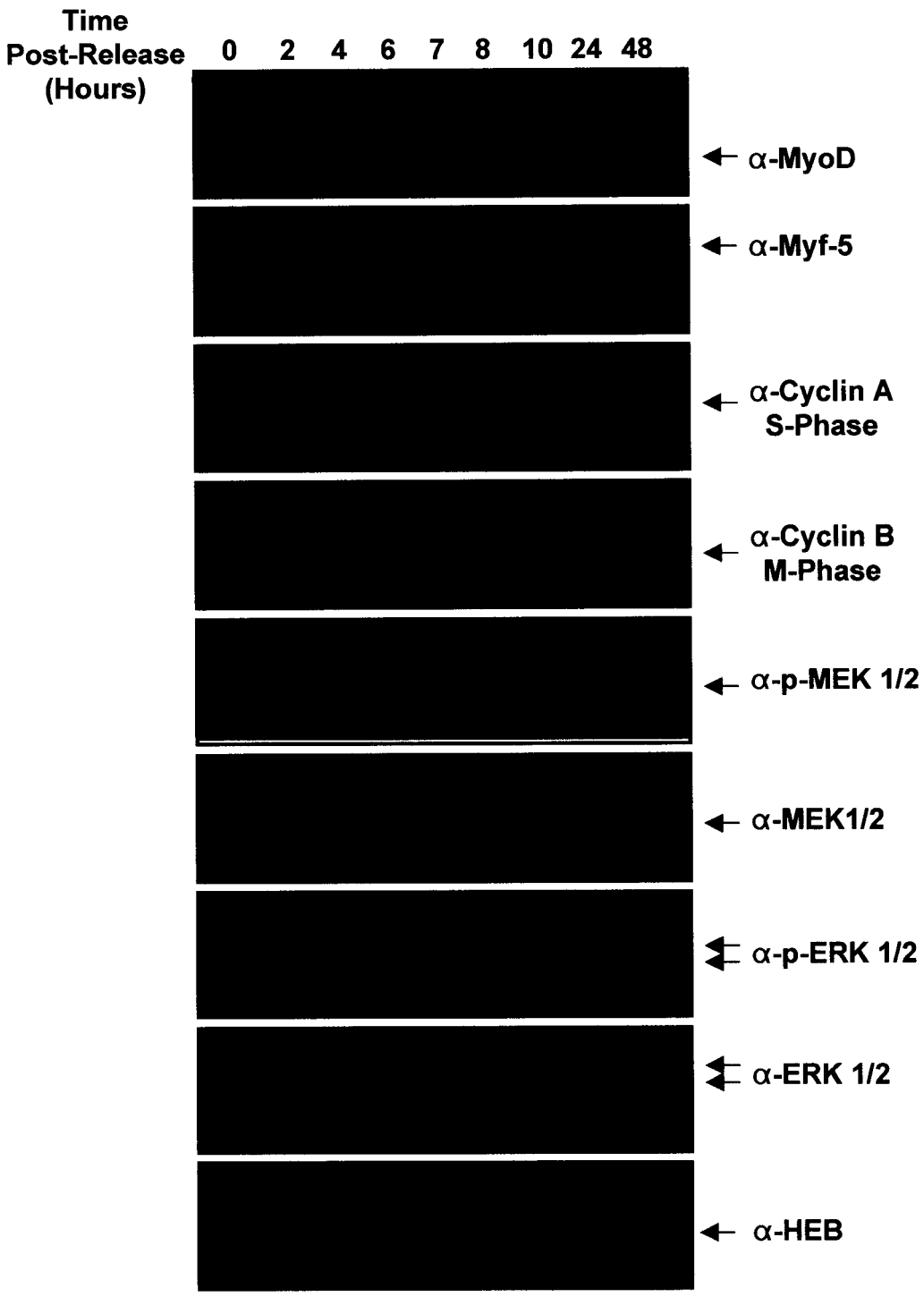

Time=9½ hrs
Time=10 hrs

Myf5 oscillate during the cell cycle, with a slight increase in the timing of these changes. Increases in cyclin A, which is necessary for the S-phase, and cyclin B, a necessary component of the mitosis promoting factor (MPF), clearly show the cells are highly synchronized with the expression of these molecules being tightly regulated (Figure 4.16). Examination of MAPK activation shows biphasic MEK and ERK activation without changes in the expression levels of the kinases (Figure 4.16; compare blots probed with p-MEK1/2 with MEK1/2 and p-ERK1/2 with ERK1/2). As predicted, MEK1 activation occurs during the G2-M transition of the cell cycle (refer to Figure 4.15B). This increase corresponds to the same phase as when MyoD levels are increasing. This suggests the potential that MEK1 activity at this stage is involved in repressing MyoD-mediated gene expression. Surprisingly, the expression pattern of the MRF dimerization partner, HEB, shows it is absent during the G1-S through the G2-M phases of the cell cycle (Figure 4.16; bottom HEB panel). The significance of this will be discussed below.

To understand the potential importance of the relationship of MyoD protein level oscillations with MEK activation, synchronized C2C12 myoblasts were released into either growth or differentiation medium in the presence or absence of the MEK inhibitor U0126 (Figure 4.17). It can be seen that inclusion of U0126 into the medium of synchronized C2C12 myoblasts released into growth medium did not result in an increase in the number of MF20-positive myotubes (Figure 4.17 A). This confirms data showing that culturing asynchronous C2C12 cells with U0126 does not increase the rate of differentiation under growth promoting conditions (data not shown). By contrast, inclusion of U0126 in the medium of C2C12 cells released under differentiating

Figure 4.16: Synchronized C2C12 myoblasts and cell cycle immunoblotting.

Synchronized C2C12 myoblasts were harvested at the times (in hours) specified after hydroxyurea release into growth medium. Immunoblots were generated using 25 μg of protein extract per lane and blots were probed as indicated. Expression profiles of MyoD and Myf5 were as predicted with a shift to a modestly longer cell cycle under the conditions used. Expression of cyclins A and B demonstrate the cells are transiting through the distinct cell cycle phases in a synchronous manner. Increased MEK activation, as determined using the phospho-specific MEK antibody, occurs at the G2-M phase of the cell cycle without alterations in the protein levels (compare p-MEK1/2 with MEK1/2). Similarly, ERK activation mimics MEK activation without changes in total protein levels (compare p-ERK1/2 with ERK1/2). Surprisingly, the levels of HEB are dramatically altered in the early phases with levels of expression barely detectable. As cells return to the G1-phase of the cell cycle, HEB is detectable. This suggests an as yet known level of regulation of myogenesis which specifically targets the MRF dimerization partner for destruction during a crucial time of the cell cycle.


Time
Post-Release
(Hours)
0 2 4 6 7 8 10 24 48
α-MyoD
α-Myf-5
α-Cyclin A
S-Phase
α-Cyclin B
M-Phase
α-p-MEK 1/2
α-MEK1/2
α-p-ERK 1/2
α-ERK 1/2
α-HEB

conditions results in a dramatic increase in the detection of terminally differentiated, MF20-positive myotubes (Figure 4.17B). Importantly, the increase in differentiation was only observed after cells were cultured for 48 hours under differentiation conditions in the presence of U0126, suggesting the cells complete one round of division from G1-S release (Figure 4.17B; compare 24- and 48-hours, +U0126). Significantly, as observed with C2C12 cells cultured for 5 days under differentiating conditions in the presence of U0126 (Figure 4.13), the number of total nuclei found per microscope field is reduced when comparing +/- U0126 suggesting MEK inhibition leads to an increase in C2C12 cell cycle exit.

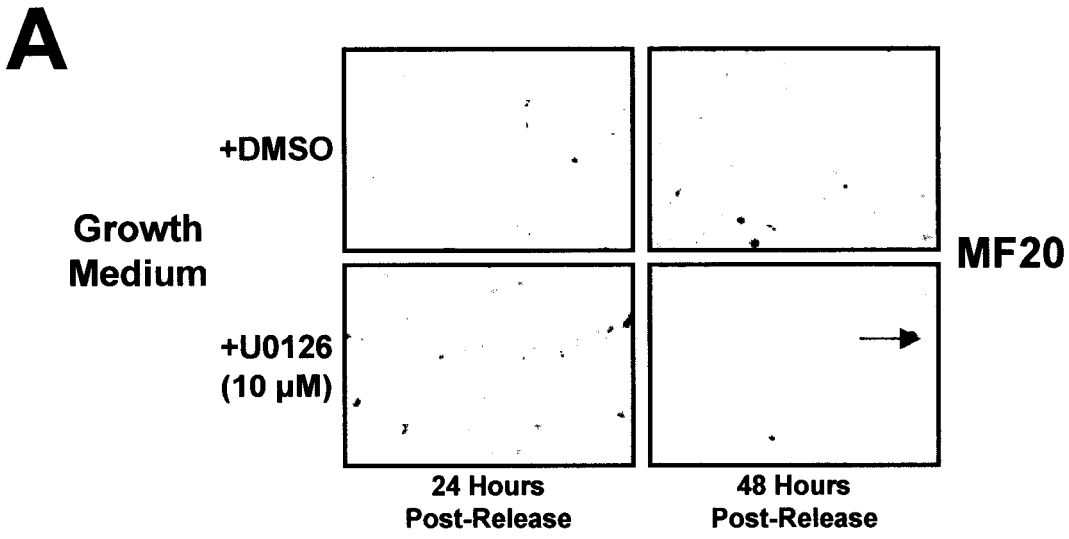
Importance of HEB regulation during the myoblast cell cycle

Necessary for the function of the MRFs is the dimerization with E-proteins. Several reports have shown that one mechanism regulating MRF-mediated gene expression is changes in the availability of E-proteins for dimerization. Data presented in the last section dealing with cell cycle progression of C2C12 myoblasts clearly shows the loss of HEB protein during the G1-S through G2-M phases of the cell cycle (Figure 4.16; lower panel). To understand the importance of this result, 10T1/2 fibroblasts were exposed to the same synchronization protocol and protein extracts were collected at time points post-release (Figure 4.18). As with C2C12 myoblasts, activation of MEK and ERK occurs at similar times post-release (compare p-MEK1/2 and p-ERK1/2 panels from Figures 4.16 and 4.18). Similarly, the expression profiles of cyclins A and B indicate that the fibroblasts are synchronized. Surprisingly, immunoblotting for HEB expression reveals that the protein is detected at all phases of the cell cycle (Figure 4.18; bottom panel). This suggests the loss of HEB expression during the cell cycle is myoblast-

Figure 4.17: Inhibition of MEK activity enhances terminal differentiation of synchronized C2C12 myoblasts.

(A) Synchronized C2C12 myoblasts were released into growth medium containing DMSO (control) or the MEK inhibitor U0126 (10 μM). Cells were fixed and processed for MF20 immunocytochemistry at 24 and 48 hours post release. Under these conditions, the inclusion of U0126 did not alter the number of MF20 positive myotubes at either time point (arrow shows one differentiated cell). Cells were counterstained with hematoxylin (purple staining) and photomicrographs were obtained using a 20X objective.

(B) Synchronized C2C12 myoblasts were released into differentiation medium containing DMSO (control) or the MEK inhibitor U0126 (10 μM). Cells were fixed and processed for MF20 immunocytochemistry at 24 and 48 hours post release. Under these conditions, the inclusion of U0126 dramatically increased the percentage of MF20 positive myotubes (brown staining; arrows) at both time points examined. Percent MF20 positive calculations represent mean values calculated using counts of more than 1000 nuclei on multiple experimental culture dishes and are as follows: DMSO 24 hours, 0.2%: U0126 24 hours, 2.19%: DMSO 48 hours, 3.55%: U0126 48 hours, 22.11%. Photomicrographs were obtained using a 20X objective.


A
+DMSO
Growth
Medium
+U0126
(10 µM)
MF20
24 Hours
Post-Release
48 Hours
Post-Release

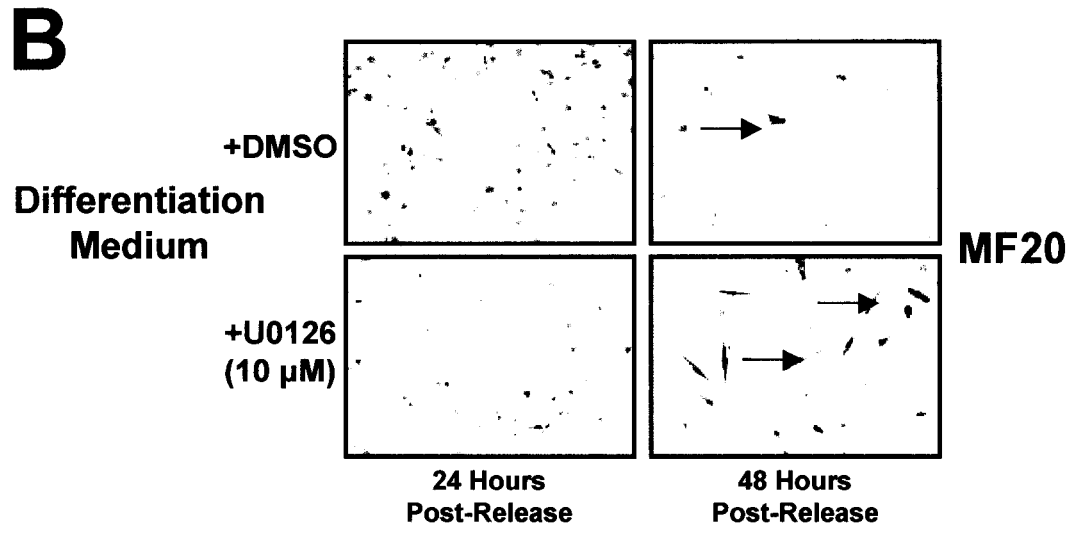

B
+DMSO
Differentiation
Medium
+U0126
(10 µM)
MF20
24 Hours
Post-Release
48 Hours
Post-Release

Figure 4.18: C3H10T1/2 fibroblast synchronization reveals muscle-specific HEB regulation.

10T1/2 fibroblasts were synchronized according to the protocol used for C2C12 myoblasts. Cell extracts were collected at the time points indicated (in hours), and immunoblots were generated using 25 μg of protein per lane and probed as indicated. Similar to C2C12 myoblasts, MEK and ERK are activated at similar times during the cell cycle without changes in expression levels. Cyclin A and B expression indicate the fibroblasts cycle with similar kinetics to C2C12 myoblasts. Interestingly, unlike myoblasts, HEB levels remain stable throughout the cell cycle. This suggests cell cycle-dependent regulation of the MRF dimerization partner is specific for skeletal muscle cells and may represent an important level of myogenic regulation.

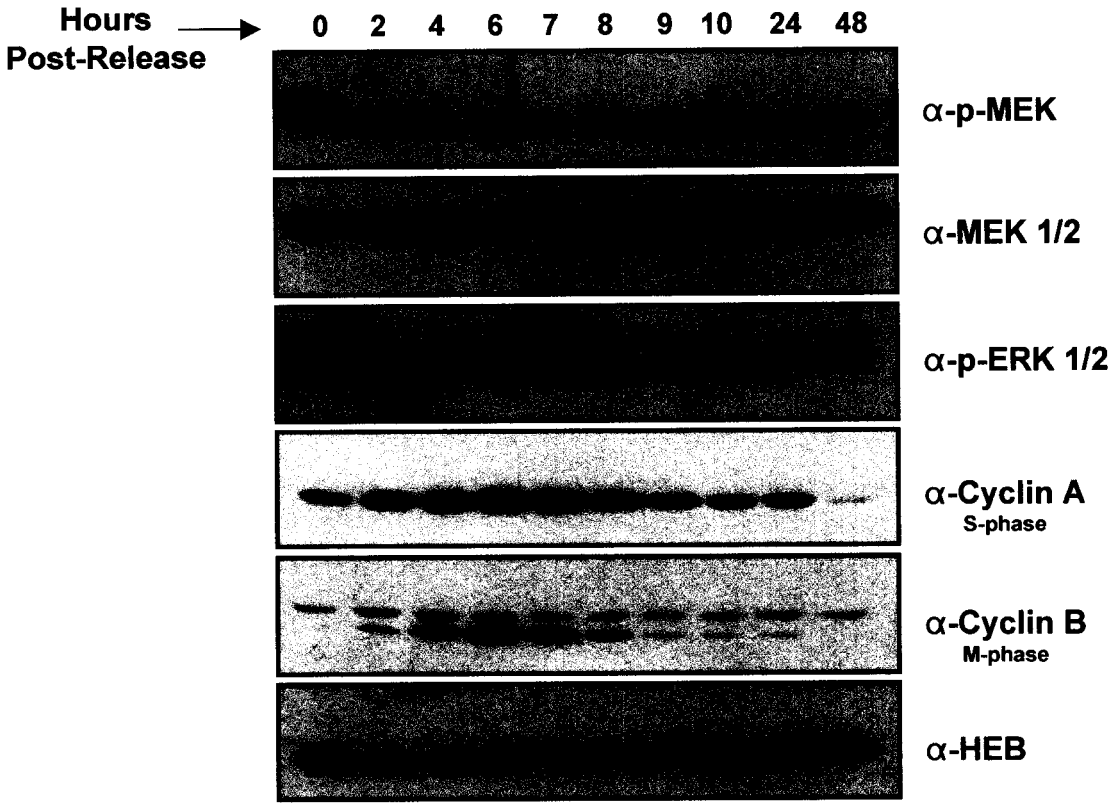
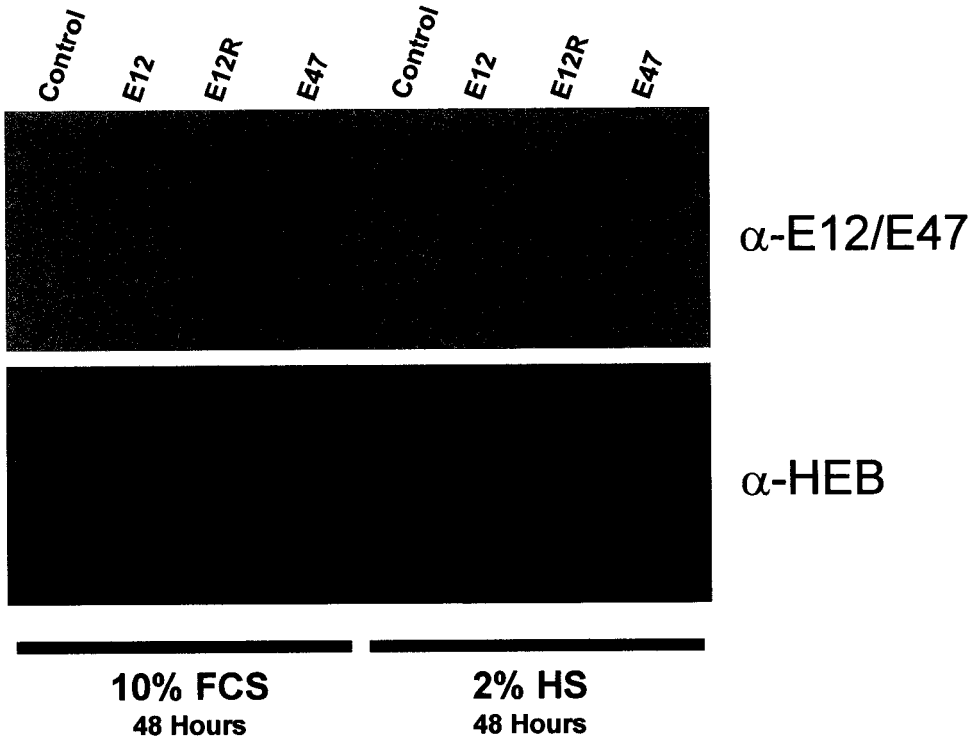

Hours
Post-Release
0 2 4 6 7 8 9 10 24 48
α-p-MEK
α-MEK 1/2
α-p-ERK 1/2
α-Cyclin A
S-phase
α-Cyclin B
M-phase
α-HEB

Figure 4.19: E-protein expression in 10T1/2 fibroblasts.

Heterodimerization of the MRFs with the ubiquitously expressed E-proteins is required for muscle-specific gene activation. To confirm that E12 and E47 were not detectable in 10T1/2 fibroblasts, cells were transfected with expression plasmids encoding E12, E12R (an N-terminal truncated mutant of E12) or E47. Cells were maintained for 48 hours under growth or differentiating. Immunoblots were generated using 50 μg of protein extracts and probed as indicated. In the absence of exogenous expression, E12/E47 are not detected under growth or differentiating conditions whereas expression of the endogenous HEB protein remains unchanged under both conditions. As with data from C2C12 myoblasts, HEB is likely the dimerization partner for MRFs as E12/E47 are not detected. It is unclear why the HEB antibody recognizes the truncated form of E12 and not the full-length versions of E12 or E47.


Control
E12
E12R
E47
Control
E12
E12R
E47
α-E12/E47
α-HEB
10% FCS
48 Hours
2% HS
48 Hours

specific. While the MRFs can dimerize with several E-protein members for activating gene expression, the changes in HEB levels are significant since expression of E12 or E47 are not detected in 10T1/2 fibroblasts unless plasmids encoding these molecules are transfected into the fibroblasts (Figure 4.19). This lack of E12/E47 proteins has also been observed from analyses of C2C12 myoblasts (data not shown). The finding that HEB is regulated during the cell cycle represents a novel mechanism for regulating myogenesis.

4.3 DISCUSSION

In the presence of several known extracellular growth factors, myoblasts are maintained in a proliferative state and are unable to exit the cell cycle and activate the myogenic program even though they express MyoD and/or Myf5. The MAPK pathway is important for mediating cellular responses to growth factor stimulation and previous reports examining the role of this pathway during myogenesis have suggested contradictory modes of action. For example, that MEK1 was important for stimulating myogenesis (Gredinger *et al.*, 1998), that MEK1 did not influence myogenesis (Ramrocki *et al.*, 1997), or alternatively, that MEK1 is important for mediating the repressive effect of growth factor stimulation on myogenesis (Dorman and Johnson, 1999; Weyman and Wolfman, 1998). In this chapter, the role of MAPK signaling was investigated and strong evidence for the functioning of this pathway during myogenic differentiation was presented.

Cotransfection studies revealed that activated MEK1 repressed MyoD activity both in transient assays and in myogenic conversion assays, and that inhibition of MEK1 promoted MyoD-mediated gene expression and precocious differentiation. Moreover, MAPK signaling is required for fusion as observed in other reports (Bennett and Tonks,

1997). However, it should be noted that U0126 has also been shown to repress MEK5/ERK5 signaling (Kamakura *et al.*, 1999) which may affect MEF2-mediated gene activation (Kato *et al.*, 1997). Although MAPKs generally affect transcription factor activity by direct phosphorylation, IP-kinase assays demonstrated that neither ERKs nor JNKs recognized MyoD as a kinase substrate. Furthermore, since the stability of MyoD protein was unaffected in the presence of a constitutively active MAPK pathway, it is unlikely that MEK1 regulation involves increasing the activity of cyclin/cdk complexes that are known to phosphorylate and target MyoD for ubiquitin-mediated degradation (Kitzmann *et al.*, 1999; Reynaud *et al.*, 1999; Song *et al.*, 1998; Tintignac *et al.*, 2000; Zhang *et al.*, 1999b). Taken together, these data suggest that signaling through MEK1 represses the ability of MyoD to transactivate differentiation-specific target genes via some novel mechanism.

Important to the functioning of MyoD are distinct domains important for nuclear localization, dimerization, chromatin-remodeling and transcriptional activity (Gerber *et al.*, 1997; Heller and Bengal, 1998; Puri *et al.*, 1997a; Puri *et al.*, 1997b; Sartorelli *et al.*, 1997; Sartorelli *et al.*, 1999; Vandromme *et al.*, 1995). The data presented here clearly demonstrated that the subcellular distribution of MyoD was unaffected by activated MEK1 confirming previous data showing MyoD remains nuclear in proliferating myoblasts (Tapscott *et al.*, 1988). An important aspect of the repressive effect of MEK1 appears to be the nuclear localization that follows activation. Physiologically, increased MEK1 nuclear translocation normally occurs following serum stimulation, at the G2-M phase of the cell cycle, and following inhibition of the nuclear export machinery (Fukuda *et al.*, 1997a; Fukuda *et al.*, 1997b; Jaaro *et al.*, 1997; Tolwinski *et al.*, 1999). Therefore,

the constitutive nuclear translocation of MEK1 ΔN3 S218E/S222D more closely mimics the normal MEK1 protein following activation and during the G2-M transition of the cell cycle. By contrast, other versions of activated MEK1 are constitutively cytoplasmic unless TPA stimulated, providing a possible explanation of differences between these results and those of other laboratories (Ramrocki *et al.*, 1997). This also suggests the repression is not ERK-mediated since these full-length forms do not repress MRF-mediated gene expression. The data presented here indicates that activated MEK1 plays a role in repressing the transcriptional activity of the MRFs.

Taken together, these data provide strong evidence that MAPK signaling negatively regulates the switch from proliferation to differentiation of myogenic cells. Interestingly, activated MEK1 becomes nuclear localized during the G2-M phase of the cell cycle (Tolwinski *et al.*, 1999). Moreover, MyoD protein levels oscillate during the cell cycle with peak values as cells approach the G2-M phase (Kitzmann *et al.*, 1998). Although the function of MEK1 nuclear translocation at the G2-M boundary remains unknown, it is interesting to speculate that in myoblasts the regulation of MyoD activity by MEK1 acts as a switch between proliferation and terminal differentiation. Indeed, inhibition of MEK activation leads to an increased rate of differentiation. Consistent with this hypothesis, treatment of terminally differentiated C2C12 myotubes with TPA did not induce nuclear translocation of MEK1.

Of great significance is the finding that the MRF dimerization partner, HEB, is not detectable during the G1-S to G2-M phases of the cell cycle. Comparison between myogenic and nonmyogenic cell types suggests this is a muscle-specific mode of

regulation. Determining whether this change in HEB stability occurs just prior to cell cycle exit and terminal differentiation is of great importance.

CHAPTER 5

MECHANISM OF MEK1 REGULATION OF MYOD ACTIVITY

5.1 INTRODUCTION

To date, several reports suggest regulation of MRF activity occurs via diverse mechanisms. These include direct protein-protein interactions (Bengal *et al.*, 1992; Li *et al.*, 1992b), posttranslational modification of critical amino acid residues regulating DNA binding (Li *et al.*, 1992a; Sartorelli *et al.*, 1999; Polesskaya *et al.*, 2000), changes in the availability of dimerization partners (Neuhold and Wold, 1993; Amy Chen *et al.*, 1996; Lu *et al.*, 1999), and alterations in the recruitment of factors necessary for chromatin remodeling and MRF-mediated transcriptional activation (Puri *et al.*, 1997a; Sartorelli *et al.*, 1997; Puri *et al.*, 1997b). Similarly, studies closely examining the intramolecular details of the MRFs have mapped several domains that serve distinct functions for MRF-mediated gene expression (Weintraub *et al.*, 1991; Gerber *et al.*, 1997; Schwarz *et al.*, 1992).

As detailed in the introduction, the basic helix-loop-helix domains are responsible for DNA-binding and dimerization, respectively. In the case of MyoD, the N-terminal 51 amino acids have been shown to be solely responsible for transcriptional activation (Weintraub *et al.*, 1991). Similar transactivation domains have been described in both N- and C-terminal regions of myogenin (Schwarz *et al.*, 1992). Also of note, within the N- and C-terminal regions of MyoD are domains responsible for chromatin remodeling. While deletion of the chromatin remodeling domains modestly affects reporter gene activation, there is clearly a deficit in the ability of deletion mutants to activate endogenous muscle-specific genes (Gerber *et al.*, 1997). By contrast, loss of the

transactivation domain of MyoD abolishes skeletal muscle-specific gene expression but the molecule still retains the ability to convert fibroblasts to the myogenic lineage (Gerber *et al.*, 1997).

Data presented in the previous chapter clearly demonstrated that the MAPK pathway acts to negatively regulate myogenesis in a similar manner to stimulation of myoblasts with extracellular growth factors. Coexpression of activated MEK1 in both 10T1/2 fibroblasts and C2C12 myoblasts inhibited terminal differentiation, but did not affect MRF protein stability, subcellular localization, nor does MyoD represent a downstream kinase substrate for the ERK or JNK pathways. An important aspect of this regulation appears to be the fact that the activated form of MEK1 used in these experiments resides in both the cytoplasmic and nuclear compartments. Moreover, MEK1 activation, and likely nuclear translocation, during the G2-M transition suggests an important, and possibly more direct role of MEK1 in regulating MRF-mediated gene expression and terminal differentiation.

To understand the underlying molecular mechanisms involved with MEK1-mediated repression of myogenesis, a determination of which MyoD domain represented the target of MAPK regulation was undertaken. Initial experiments demonstrated that activated MEK1 regulated multiple domains of MyoD or regulated the dimerization/DNA-binding capabilities. However, substitution of the MyoD transactivation domain with the VP16 acidic transactivation domain clearly shows MAPK-mediated regulation is targeted to the N-terminal portion of MyoD. Indeed, inclusion the VP16 transactivation domain fused to the N-terminal chromatin remodeling domain and the bHLH region permitted unregulated muscle-specific reporter gene

activation and, unaffected activation of endogenous, myogenic gene targets. Surprisingly, coimmunoprecipitation experiments showed that activated MEK1 interacts with a transactivator complex containing MyoD and its dimerization partner HEB. Importantly, the interaction is specific for, and requires, the N-terminal transactivation domain of MyoD. However, MEK1 binding to the MyoD/HEB transcriptional complex appears to require at least one other cofactor, likely specific to myogenic cells. The data presented in this chapter clearly demonstrate a mechanism by which MAPK signaling can repress myogenesis and maintain cells in a proliferative state without a concomitant loss of myogenic identity due to MRF degradation.

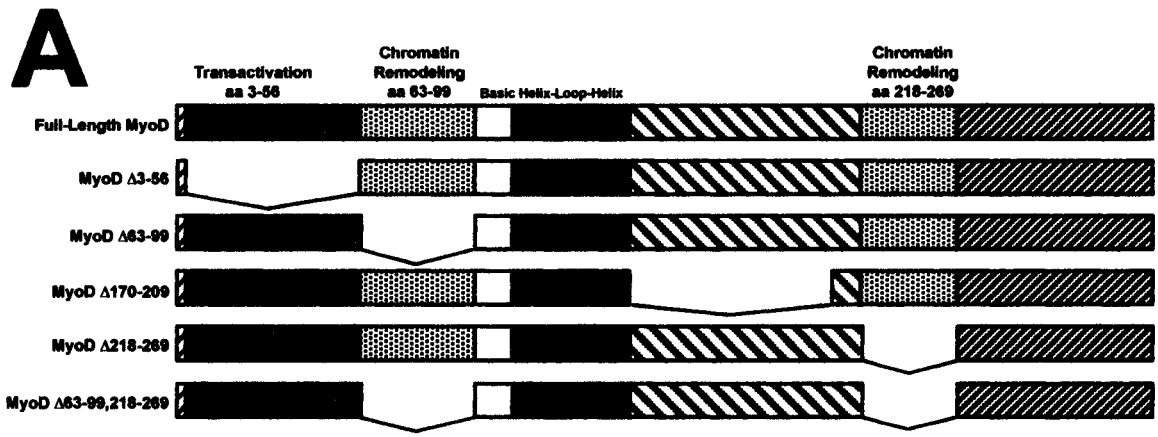
5.3 RESULTS

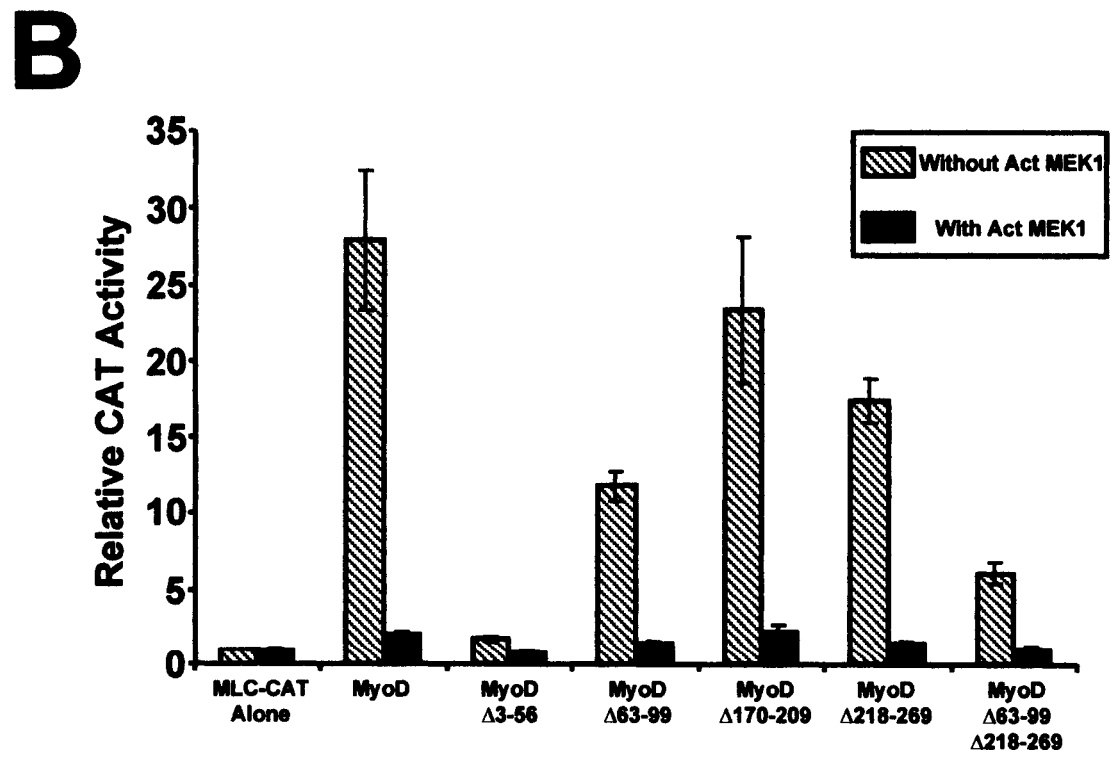
Important to the function of the MRFs are distinct domains required for dimerization, DNA binding, phosphorylation and protein-protein interactions (for review see Puri and Sartorelli, 2000). Molecular dissection and functional studies of distinct domains of the MRFs have clearly demonstrated the existence of separable elements necessary for transcriptional activation and chromatin remodeling (Weintraub *et al.*, 1991; Gerber *et al.*, 1997). To address if MEK1-mediated repression is targeted to a specific functional domain of MyoD, the transcriptional and conversion activities of MyoD deletion mutants were examined when coexpressed with activated MEK1. A schematic of the domains of MyoD and the deletion mutants used for these studies is shown (Figure 5.1A). To assess whether activated MEK1 targeted a specific functional domain of MyoD, the transcriptional activity of the deletion mutants was determined using the MLC-CAT reporter vector (Figure 5.1B). The different levels of transactivation in the absence of activated MEK1 are similar to that observed in other

Figure 5.1: Effect of activated MEK1 on MyoD deletion mutants.

(A) Several domains of MyoD have been shown to play a role in dimerization (helix-loop-helix), DNA binding (basic), gene activation (transactivation) and chromatin remodeling. Deletion of chromatin remodeling domains moderately affects reporter gene activation but severely affects activation of endogenous genes. By contrast, deletion of the transactivation domain abolishes gene activation but does not hinder chromatin remodeling of endogenous, muscle-specific genes. The schematic shows the domain mutants of MyoD that were used to assess the specific mode of MEK1-mediated repression of myogenesis.

(B) Coexpression of activated MEK1 with all deletion mutants of MyoD leads to a dramatic reduction of MyoD-mediated activation of the MLC-CAT reporter vector. C3H10T1/2 fibroblasts were transfected with full-length and domain-deleted mutants of MyoD in the absence or presence of activated MEK1. Cells were harvested after 48 hours under differentiating conditions and CAT assays were performed. The levels of transactivation in the absence of MEK1 are similar to previously published reports (Gerber *et al.*, 1997). Coexpression of all deletion mutants with activated MEK1 leads to a dramatic reduction in MyoD-mediated gene expression in a similar manner as seen with full-length MyoD. Due to the lack of transcriptional activity of the Δ3-56 mutant, it is difficult to assess whether activated MEK1 influences this domain.

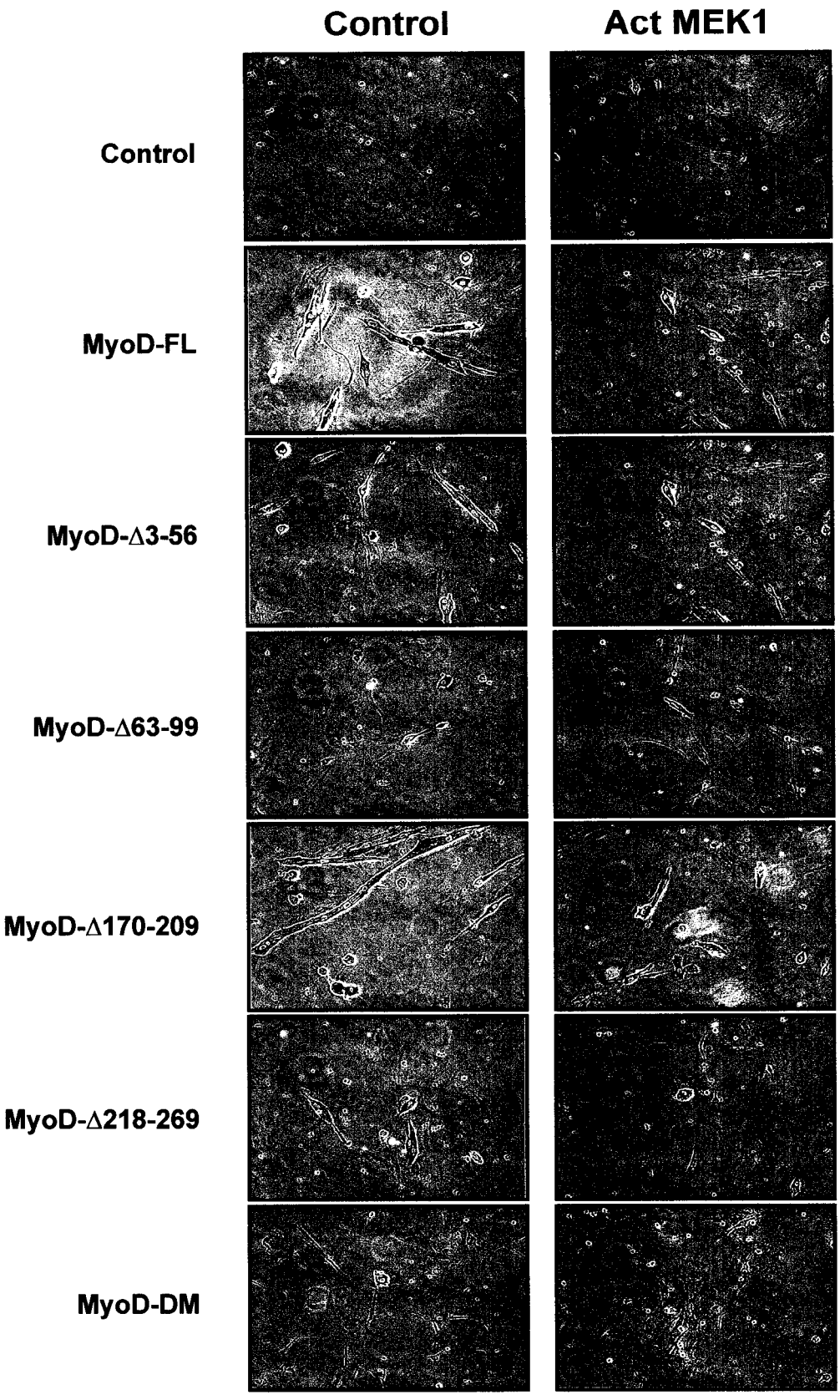

A
Transactivation
aa 3-56
Chromatin
Remodeling
aa 63-99
Basic Helix-Loop-Helix
Chromatin
Remodeling
aa 218-269
Full-Length MyoD
MyoD Δ3-56
MyoD Δ63-99
MyoD Δ170-209
MyoD Δ218-269
MyoD Δ63-99,218-269


B
Relative CAT Activity
0
5
10
15
20
25
30
35
Without Act MEK1
With Act MEK1
MLC-CAT Alone
MyoD
MyoD Δ3-56
MyoD Δ63-99
MyoD Δ170-209
MyoD Δ218-269
MyoD Δ63-99 Δ218-269

reports (Gerber *et al.*, 1997). As seen with the full-length molecule, all MyoD deletion mutants are repressed in their ability to activate transcription under differentiation conditions with a strong MAPK signal. Since the ability to transactivate and conversion are carried out by distinct domains, the ability of these MyoD mutants to convert 10T1/2 fibroblasts was also assessed (Figure 5.2). Similar to the transactivation experiment with full-length MyoD, conversion and terminal differentiation of 10T1/2 fibroblasts is compromised when activated MEK1 is coexpressed as measured by MF20 immunocytochemistry (Figure 5.2). Furthermore, the almost complete absence of conversion with the DM-MyoD (lacking both N- and C-terminal chromatin-remodeling domains) supports data showing that at least one of these domains is required for activation of the myogenic program (Gerber *et al.*, 1997). Together, this data suggests that MEK1-mediated regulation involves multiple domains or is directed at the dimerization or DNA binding domains. It should be noted that the inability of the transactivation domain (TAD) mutant to activate gene expression makes conclusions on this domain difficult.

To address the role of each domain separately in the absence of myogenic DNA-binding requirements, Gal4 DNA binding domain (Gal4DBD) fusions were produced. The Gal4 DBD is from a yeast transcription factor that forms very stable dimers that bind to a specific DNA sequence called the Gal4 UAS (upstream activating sequence) (Sadowski *et al.*, 1988). Fusions of the N-terminus of MyoD that include the transactivation and amino-terminal chromatin-remodelling domains are shown along with the reporter vector (Figure 5.3A). As seen with the N-terminus of MRF4 (Ramrocki *et al.*, 1997), transcriptional activity mediated by the N-terminal transactivation domain is

Figure 5.2: Conversion and terminal differentiation is reduced by activated MEK1. The ability of MyoD deletion mutants to convert 10T1/2 fibroblasts was assessed by MF20 immunocytochemistry after 48 hours in differentiation medium. As observed for transactivation, coexpression of activated MEK1 prevents MyoD deletion mutants to convert 10T1/2 fibroblasts to the myogenic lineage. It can be seen that deletion of the chromatin-remodeling domains (Δ63-99, Δ218-269, and the double mutant (DM) that lacks both chromatin-remodeling domains) severely reduces the converting potential of MyoD whereas deletion of the TAD only leads to a moderate reduction in conversion of 10T1/2 fibroblasts to the myogenic lineage.

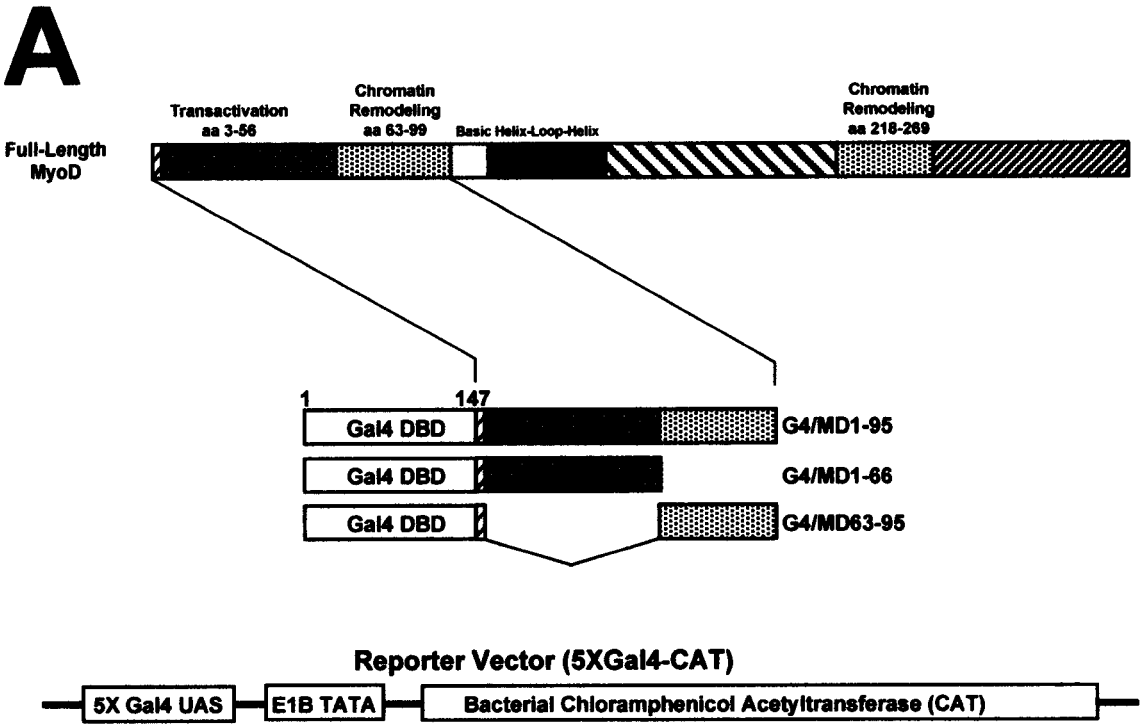


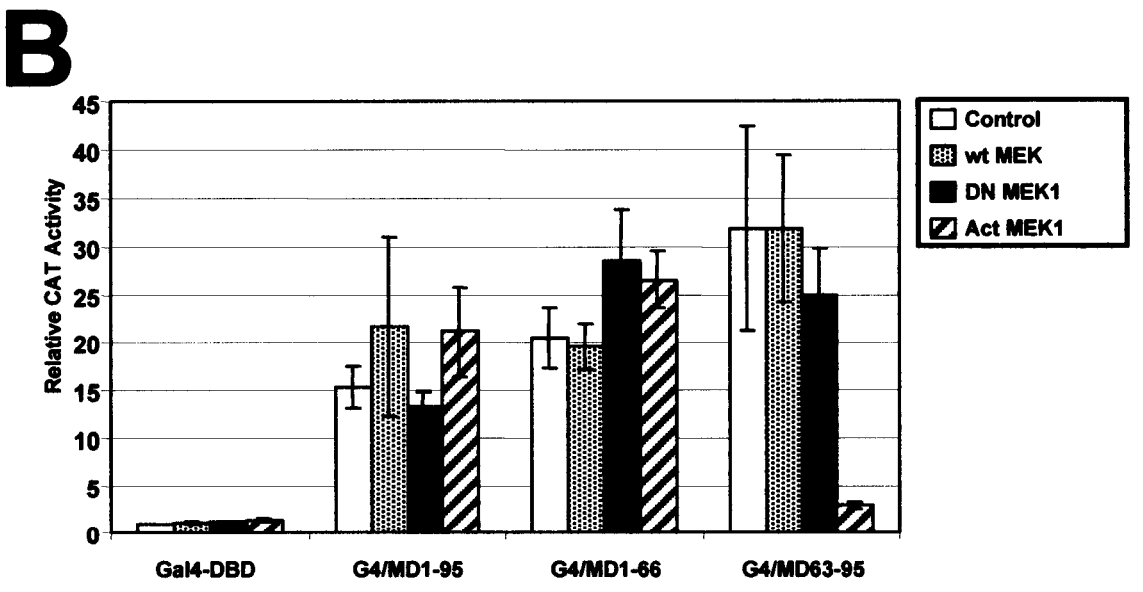
5 Days Differentiation
MF20 Immunocytochemistry

Figure 5.3: Activated MEK does not inhibit inherent transactivation of the amino-terminal portion of MyoD.

(A) Schematic diagram of the N-terminal fusions of MyoD to the Gal4 DNA-binding domain (Gal4 DBD). Also shown is the reporter vector used to assess transcriptional activity.

(B) Coexpression of activated MEK1 does not inhibit transcriptional activity of Gal4 DBD fusions that include the acidic transactivation domain of MyoD. Surprisingly, the N-terminal chromatin-remodeling domain demonstrates inherent transactivation ability. Coexpression of activated MEK1 represses the transcriptional activity of G4/MD63-95. Transfected fibroblasts were harvested after 48 hours in differentiation medium and CAT assays were performed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=9).


A
Transactivation
aa 3-56
Chromatin
Remodeling
aa 63-99
Basic Helix-Loop-Helix
Chromatin
Remodeling
aa 218-269
Full-Length
MyoD
1
147
Gal4 DBD
G4/MD1-95
Gal4 DBD
G4/MD1-66
Gal4 DBD
G4/MD63-95
Reporter Vector (5XGal4-CAT)
5X Gal4 UAS
E1B TATA
Bacterial Chloramphenicol Acetyltransferase (CAT)


B
Relative CAT Activity
45
40
35
30
25
20
15
10
5
0
Gal4-DBD
G4/MD1-95
G4/MD1-66
G4/MD63-95
Control
wt MEK
DN MEK1
Act MEK1

unaffected by coexpression of activated MEK1 (Figure 5.3B). Unexpectedly, the chromatin-remodeling domain demonstrated a high level of inherent transcriptional activity. However, coexpression of MEK1 completely represses transcriptional activation (Figure 5.3B).

Similar experiments were carried out examining the C-terminal regions of MyoD fused to the Gal4DBD (Figure 5.4A). Contrary to previously published reports (Weintraub *et al.*, 1991; Gerber *et al.*, 1997), essentially all C-terminal regions of MyoD demonstrate high levels of inherent transactivation ability (Figure 5.4 B & C). This result is similar to data obtained examining the functional domains of myogenin (Schwarz *et al.*, 1992). With the exception of the G4/MD174-269 fusion protein, coexpression of activated MEK1 negatively affects the transactivation ability of the C-terminal Gal4DBD fusions (Figure 5.4 B & C). Together, the data suggests that activated MEK1 likely affects DNA binding and/or dimerization as the inherent transcriptional activity of MyoD is unaffected. Importantly, activated MEK1 does not affect the transcriptional activity of a Gal4DBD fusion with the VP16 acidic transactivation domain (Figure 5.5). This indicates that Gal4 dimerization, DNA-binding or the transactivation potential of VP16 are not affected by activated MEK1. Therefore, the effects observed on the domains of MyoD are directed specifically to those regions of MyoD.

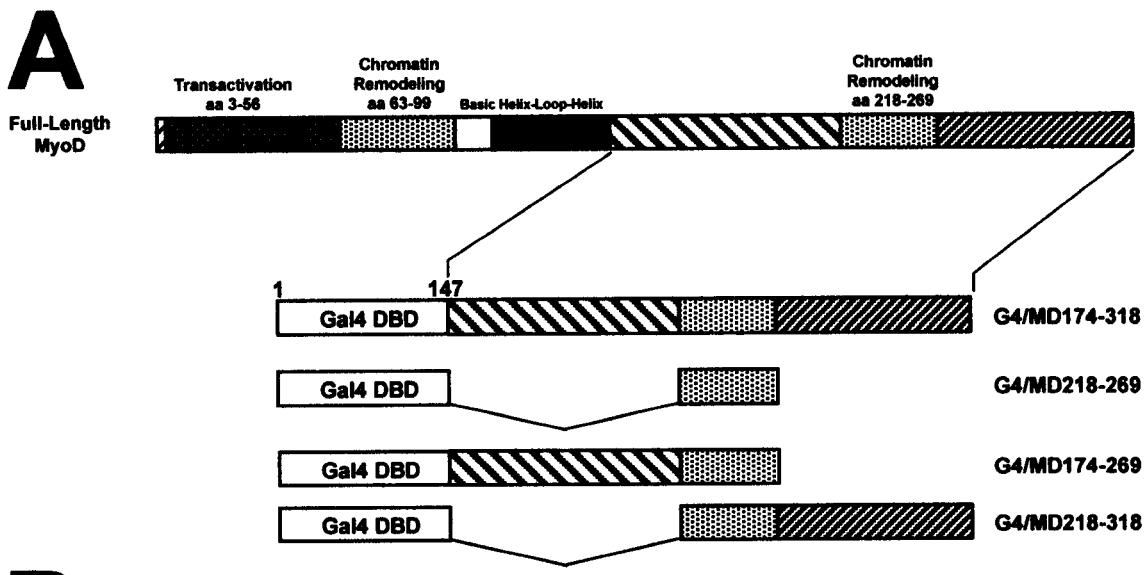
To specifically address the effects of activated MEK1 on the N-terminal transactivation and bHLH domains of MyoD, truncation mutants lacking the C-terminal portion were generated that either had the wild-type or VP16 transactivation domain, and either included or excluded the N-terminal chromatin remodeling domain (Figure 5.6A). As seen with full-length and deletion mutants of MyoD containing the N-terminal

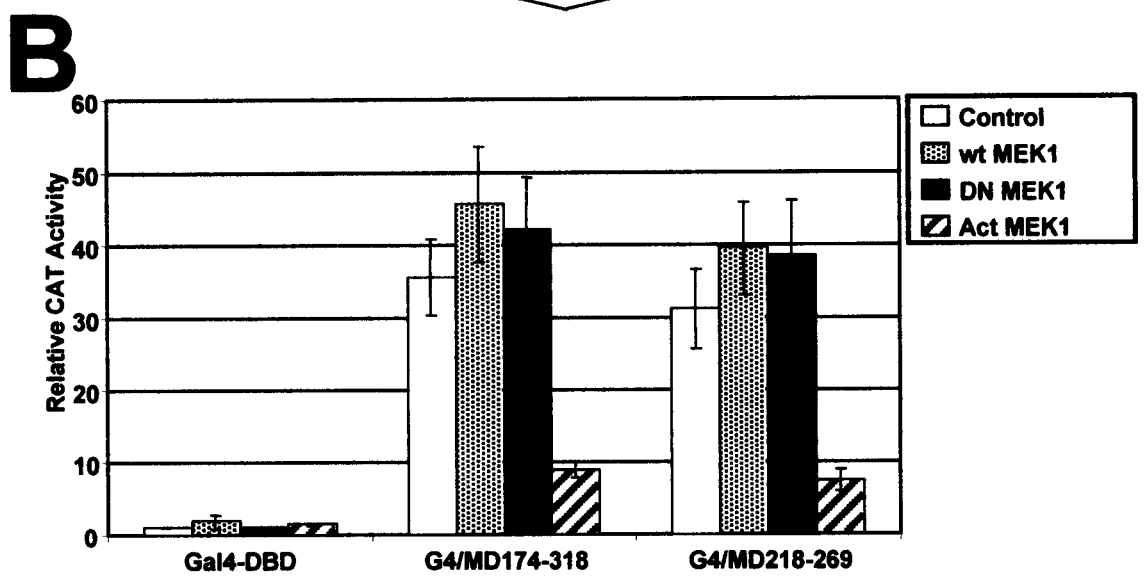
Figure 5.4: Activated MEK inhibits activation by all C-terminal regions of MyoD.

(A) Schematic diagram of the C-terminal fusions of MyoD to the Gal4 DBD.

(B) Gal4DBD fusions of the entire C-terminal (G4/MD174-318) and chromatin-remodeling (G4/MD218-269) domains show inherent transactivation ability that is repressed upon coexpression of activated MEK1. Transfected fibroblasts were harvested after 48 hours in differentiation medium and CAT assays were performed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=9).

(C) Gal4DBD fusions of C-terminal portions of MyoD demonstrate inherent transactivation ability that is repressed upon coexpression with activated MEK1. The level of MEK1-mediated repression of the G4/MD174-269 is not as complete as seen with other fusions suggesting the far C-terminal region may represent a region that responds to MEK1 regulation. Transfected fibroblasts were harvested after 48 hours in differentiation medium and CAT assays were performed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=9).


A
Full-Length MyoD
Transactivation aa 3-56
Chromatin Remodeling aa 63-99
Basic Helix-Loop-Helix
Chromatin Remodeling aa 218-269
1
147
Gal4 DBD
G4/MD174-318
Gal4 DBD
G4/MD218-269
Gal4 DBD
G4/MD174-269
Gal4 DBD
G4/MD218-318


B
Relative CAT Activity
60
50
40
30
20
10
0
Control
wt MEK1
DN MEK1
Act MEK1
Gal4-DBD
G4/MD174-318
G4/MD218-269

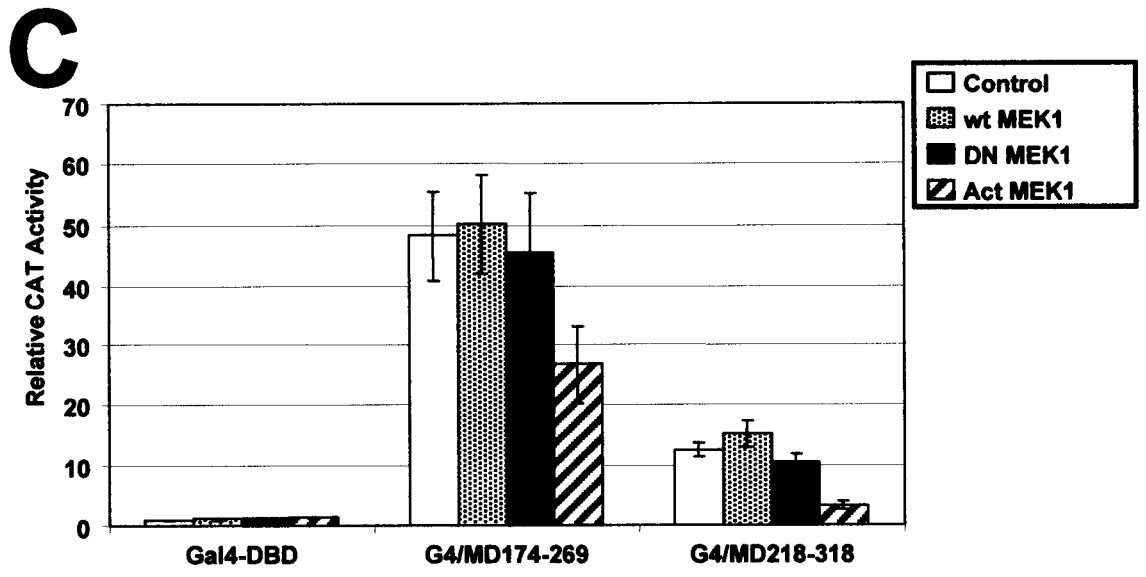
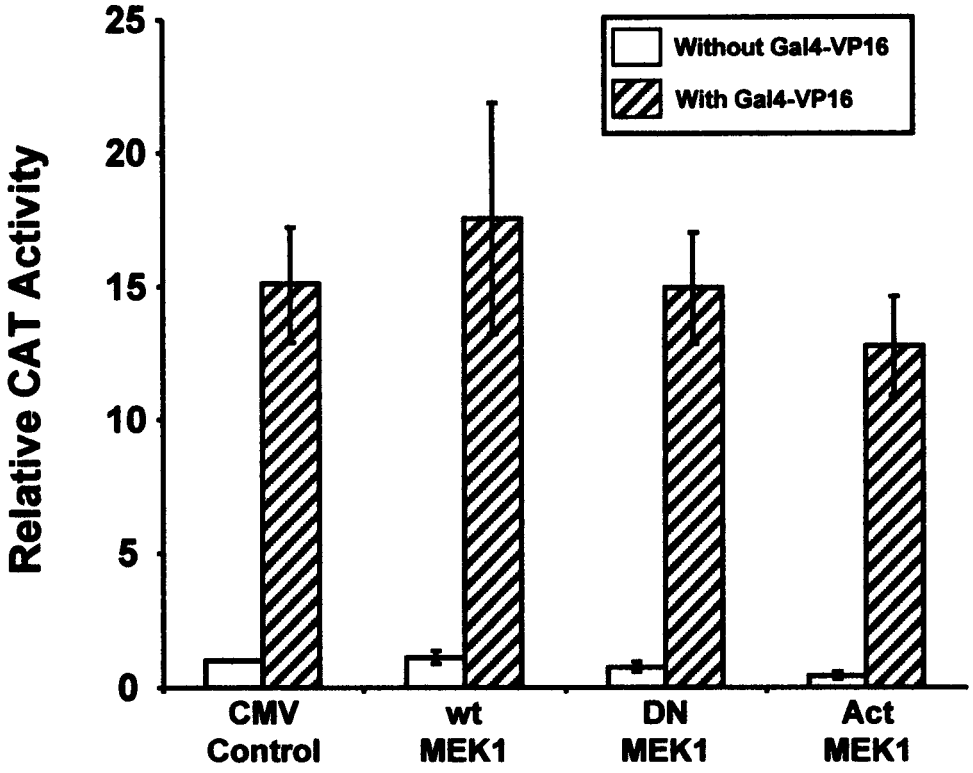

C
Relative CAT Activity
70
60
50
40
30
20
10
0
Control
wt MEK1
DN MEK1
Act MEK1
Gal4-DBD
G4/MD174-269
G4/MD218-318

Figure 5.5: Activated MEK1 does not affect the Gal4-VP16 fusion protein.

To ensure that coexpression of the MEK1 mutants does not regulate Gal4 dimerization, DNA binding or the transactivation of the VP16 acidic transactivation domain, C3H10T1/2 fibroblasts were cotransfected with Gal4-VP16, mutant MEK1 and the 5XGal4UAS-CAT vectors. Cells were harvested after 48 hours in differentiation medium and CAT assays were performed. It can be seen that transactivation is essentially equivalent irrespective of the MEK1 mutant that is expressed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=6).


Relative CAT Activity
25
20
15
10
5
0
Without Gal4-VP16
With Gal4-VP16
CMV Control
wt MEK1
DN MEK1
Act MEK1

transactivation domain, coexpression of activated MEK1 represses the ability of C-terminal truncated mutants to activate the 4RtkCAT reporter vector under differentiation conditions (Figure 5.6B). By contrast, substitution of the wild-type TAD with the VP16 TAD leads to full transcriptional activation when activated MEK1 is coexpressed (Figure 5.6C). Transactivation is unaffected with or without the N-terminal chromatin-remodeling domain, suggesting this region of MyoD is not regulated by MAPK signaling for transcriptional activation (Figure 5.6C; compare VP16bHLH with VP16-63bHLH). Furthermore, the ability of the VP16 fusions to activate the muscle-specific reporter indicates that dimerization and DNA binding are unaffected.

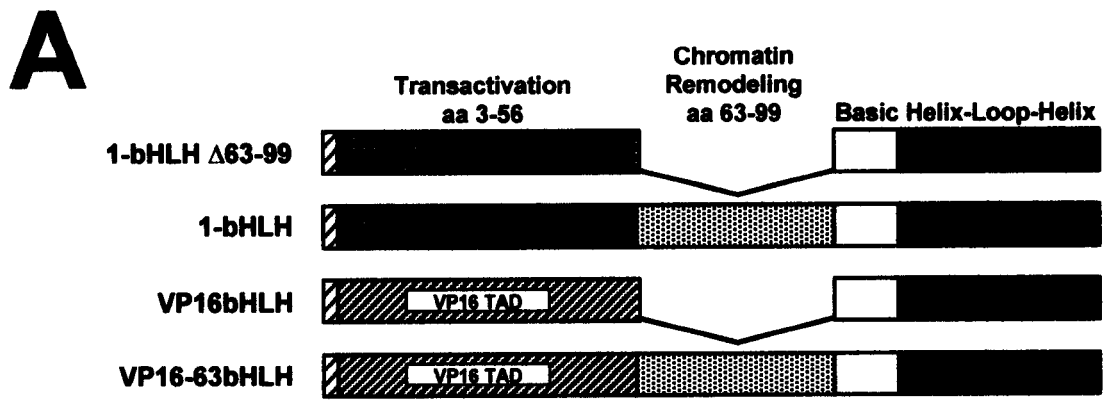
To assess the biological activity of the C-terminal truncations, activation of the myogenic program in fibroblasts was examined (Figure 5.7). Truncation mutants lacking the N-terminal chromatin-remodeling do not show an appreciable capacity for converting 10T1/2 fibroblasts to the myogenic lineage (data not shown). However, both 1-bHLH and VP16-63bHLH are competent for activation of the myogenic program as assessed by MF20 immunocytochemistry (Figure 5.7A; left panels). Coexpression of activated MEK1 essentially abolishes the ability of 1-bHLH to convert fibroblasts (Figure 5.7A; top right panel). By contrast, VP16-63bHLH remains competent for myogenic induction although myotube size is reduced (Figure 5.7A; bottom right panel). The relative level of conversion by VP16-63bHLH when coexpressed with MEK1 mutants was determined by immunoblotting of extracts from transfected cells (Figure 5.7B). It can be seen that levels of the transfected HA-tagged MEK1 mutants and VP16-63bHLH show similar levels of expression. Immunodetection of myogenin shows that expression levels are essentially identical in all lanes (Figure 5.7B; bottom panel). This indicates that VP16-

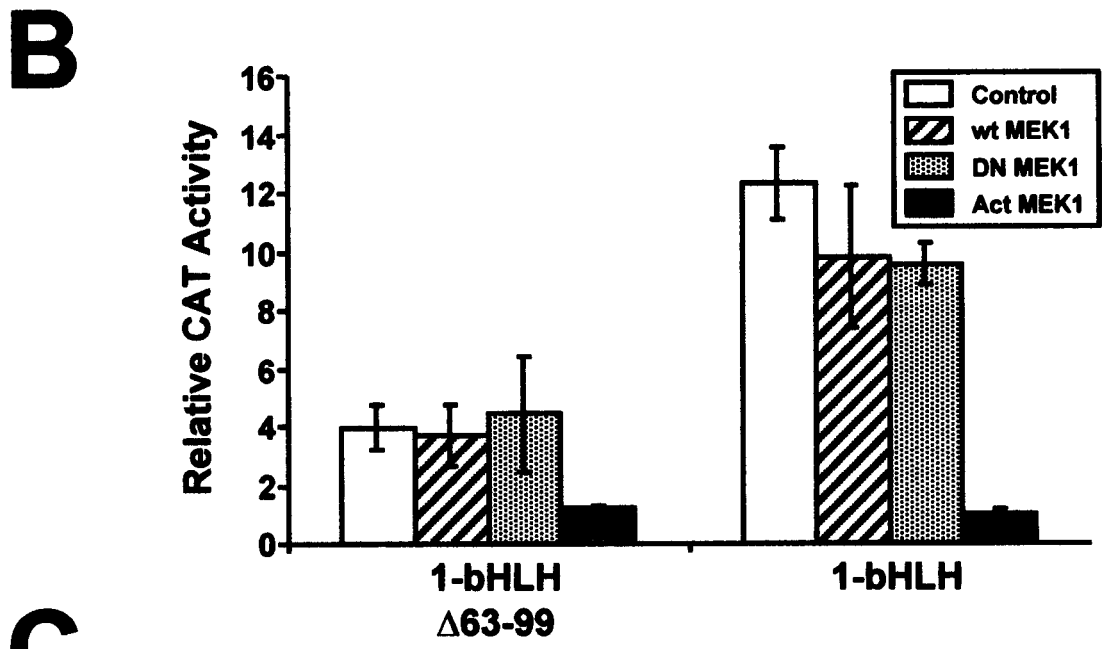
Figure 5.6: Acitvated MEK is unable to affect VP16 transactivation domain when fused to bHLH domain of MyoD.

(A) Schematic of the C-terminal truncation mutants used to examine MEK1-mediated effects on DNA-binding and dimerization. Molecules differentially include the N-terminal chromatin-remodeling domain and have the N-terminal TAD substituted with the VP16 TAD.

(B) Transfection of 10T1/2 fibroblasts with C-terminal truncation mutants containing the wild-type MyoD TAD reveals that activated MEK1 represses transcriptional activation of the 4RtkCAT reporter vector. Fibroblasts were harvested after 48 hours in differentiation medium and CAT assays were performed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=3). One representative experiment is shown.

(C) Transfection of 10T1/2 fibroblasts with VP16-containing C-terminal truncation mutants reveal that activated MEK1 is unable to repress transcriptional activity from the 4RtkCAT reporter vector. Fibroblasts were harvested after 48 hours in differentiation medium and CAT assays were performed. Bars represent the mean and error bars represent the standard error of the mean (+/- SEM; n=3). One representative experiment is shown.


A
Transactivation
aa 3-56
Chromatin
Remodeling
aa 63-99
Basic Helix-Loop-Helix
1-bHLH Δ63-99
1-bHLH
VP16bHLH
VP16 TAD
VP16-63bHLH
VP16 TAD


B
Relative CAT Activity
16
14
12
10
8
6
4
2
0
Control
wt MEK1
DN MEK1
Act MEK1
1-bHLH
Δ63-99
1-bHLH

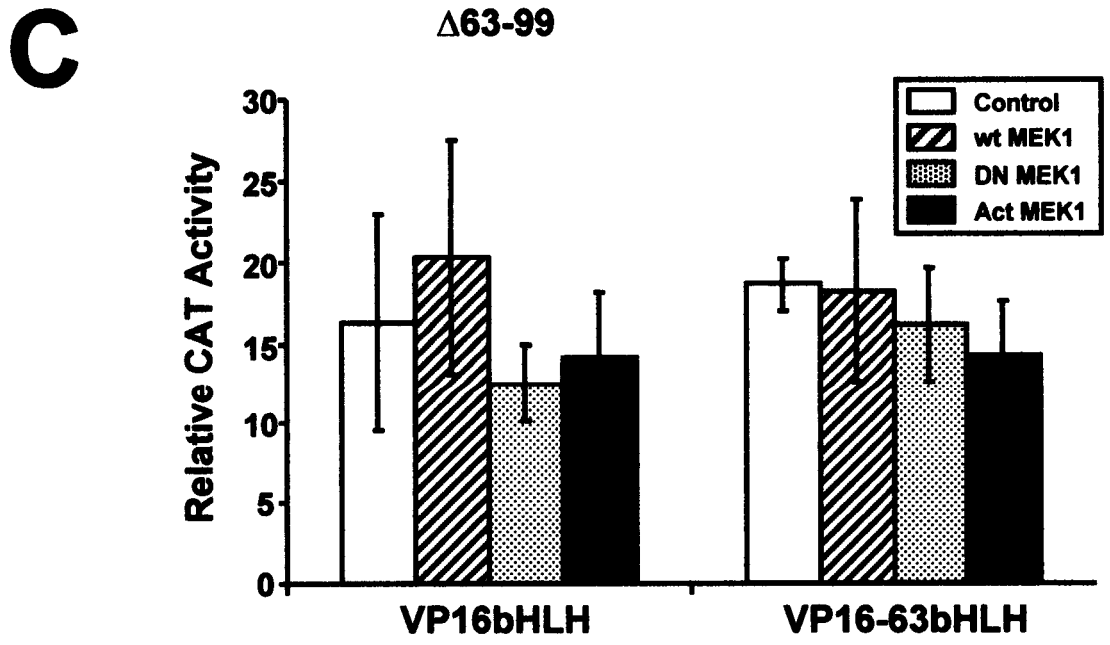
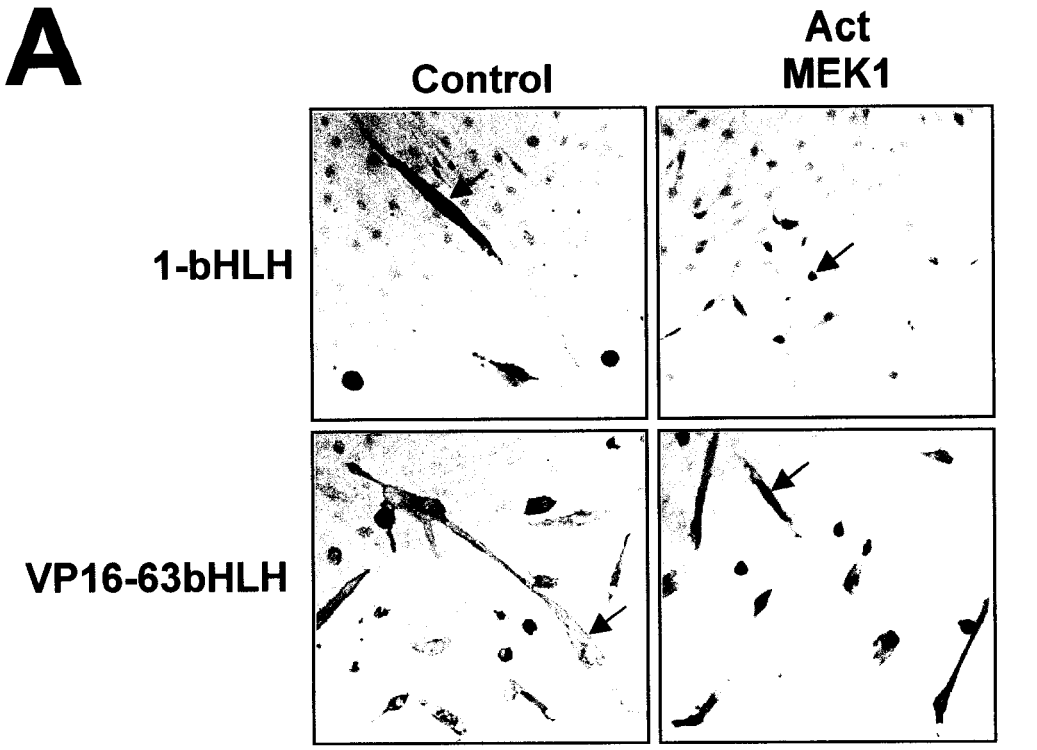
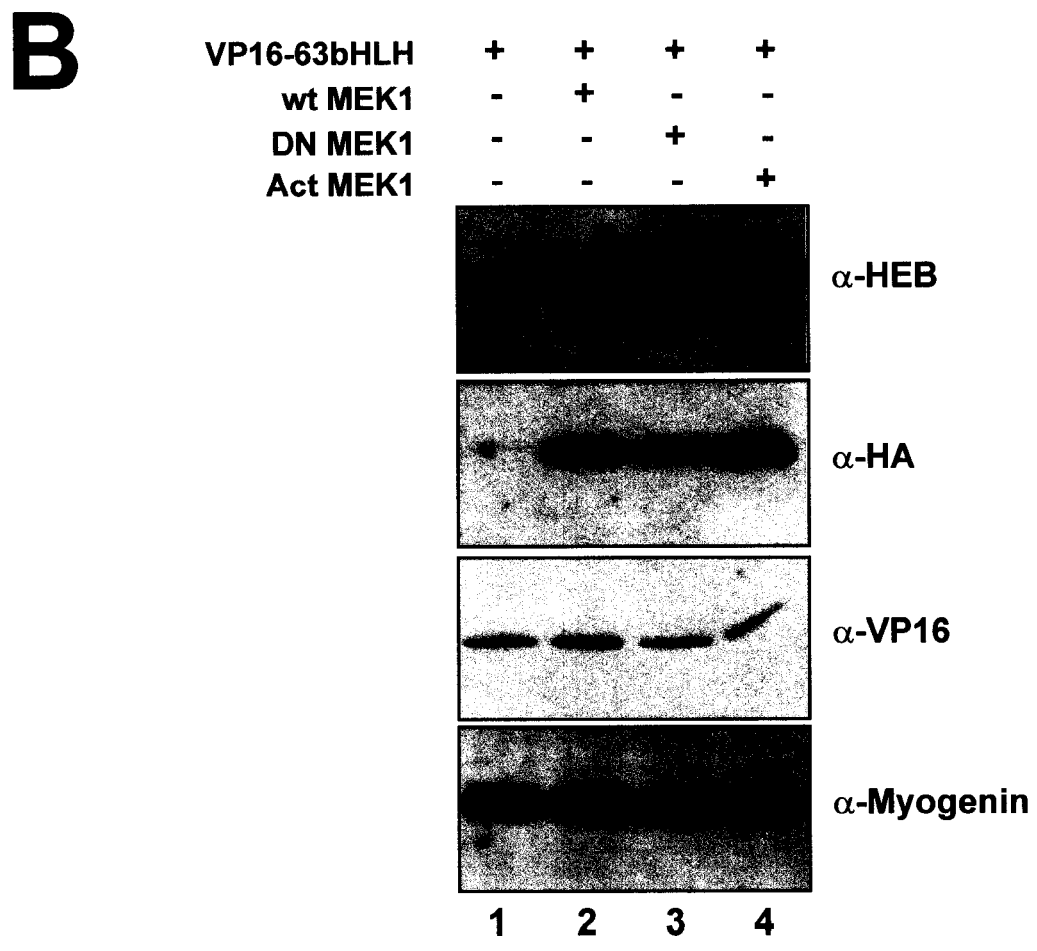

C
Relative CAT Activity
30
25
20
15
10
5
0
Control
wt MEK1
DN MEK1
Act MEK1
VP16bHLH
VP16-63bHLH

Figure 5.7: Activated MEK1 does not inhibit VP16-63bHLH-mediated activation of endogenous gene markers of myogenesis.

(A) Coexpression of activated with the C-terminal truncated mutant of MyoD dramatically reduces the ability to this factor to initiate myogenesis in 10T1/2 fibroblasts. By contrast, expression of acitvated MEK1 reduces myotube size but not efficiency of VP16-63bHLH to convert 10T1/2 fibroblasts to the myogenic lineage. Fibroblasts were transfected with the indicated plasmids and were processed for MF20 immunocytochemistry after 48 hours in differentiation medium.

(B) Immunoblot analysis of myogenin levels in 10T1/2 cells cotransfected with MEK1 mutants and VP16-63bHLH. Expression levels of HEB, MEK mutants and the VP16 fusion are essentially equivalent. Activation of myogenin is unchanged indicating that in the absence of the N-terminal transactivation domain of MyoD, myogenesis is able to proceed unaffected by the expression of activated MEK1. Fibroblasts were transfected as indicated. Cells were harvested and extracted after 48 hours in differentiation medium and immunoblots were generated using 25 μg of protein extract per lane. Blots were probed as indicated.


A
Control
Act MEK1
1-bHLH
VP16-63bHLH


B
VP16-63bHLH + + + +
wt MEK1 - + - -
DN MEK1 - - + -
Act MEK1 - - - +
α-HEB
α-HA
α-VP16
α-Myogenin
1 2 3 4

63bHLH is able to induce full activation of the myogenic program when coexpressed with activated MEK1. Taken together, this data clearly shows that the MAPK pathway does not affect dimerization and DNA binding and, within a myogenic context, the N-terminal TAD represents the target of MEK1-mediated repression of MyoD transcriptional activity.

Previous reports examining the role of the MEK1 upstream activating kinase Mos demonstrated that MyoD could interact with Mos and block Mos-mediated activation of MEK (Lenormand *et al.*, 1997; Pelpel *et al.*, 2000). This study did not address whether there was an interaction between MyoD and MEK1. To address this issue, cell extracts from 10T1/2 fibroblasts transfected with MyoD and with or without activated MEK1 were analyzed for the potential that MyoD and MEK1 are found in a multiprotein complex. As observed earlier, cells transfected with MyoD in the presence and absence of MEK1 show similar levels of expression (Figure 5.8A). When these extracts are immunoprecipitated with anti-MyoD or anti-HEB antibodies, the HA-tagged MEK1 can be found by immunoblotting (Figure 5.8B). To evaluate whether the amino-terminus was necessary for this interaction, 10T1/2 cells were transfected with full-length and deletion mutants of MyoD with activated MEK1. Similar levels of transfected proteins were detected by immunoblotting of extracts from transfected cells (Figure 5.8C). When these extracts are immunoprecipitated with an anti-MyoD or anti-HEB antibody it can be seen that in the absence of the N-terminal transactivation domain, HA-tagged MEK1 does not coimmunoprecipitate with the MyoD/HEB complex (Figure 5.8D). Indeed, GST-pull downs of cells cotransfected with full-length MyoD and activated MEK1 show that the N-terminus specifically interacts with activated MEK1 while the C-terminus does not

Figure 5.8: Interaction of MEK1 with MyoD requires the N-terminus of MyoD.

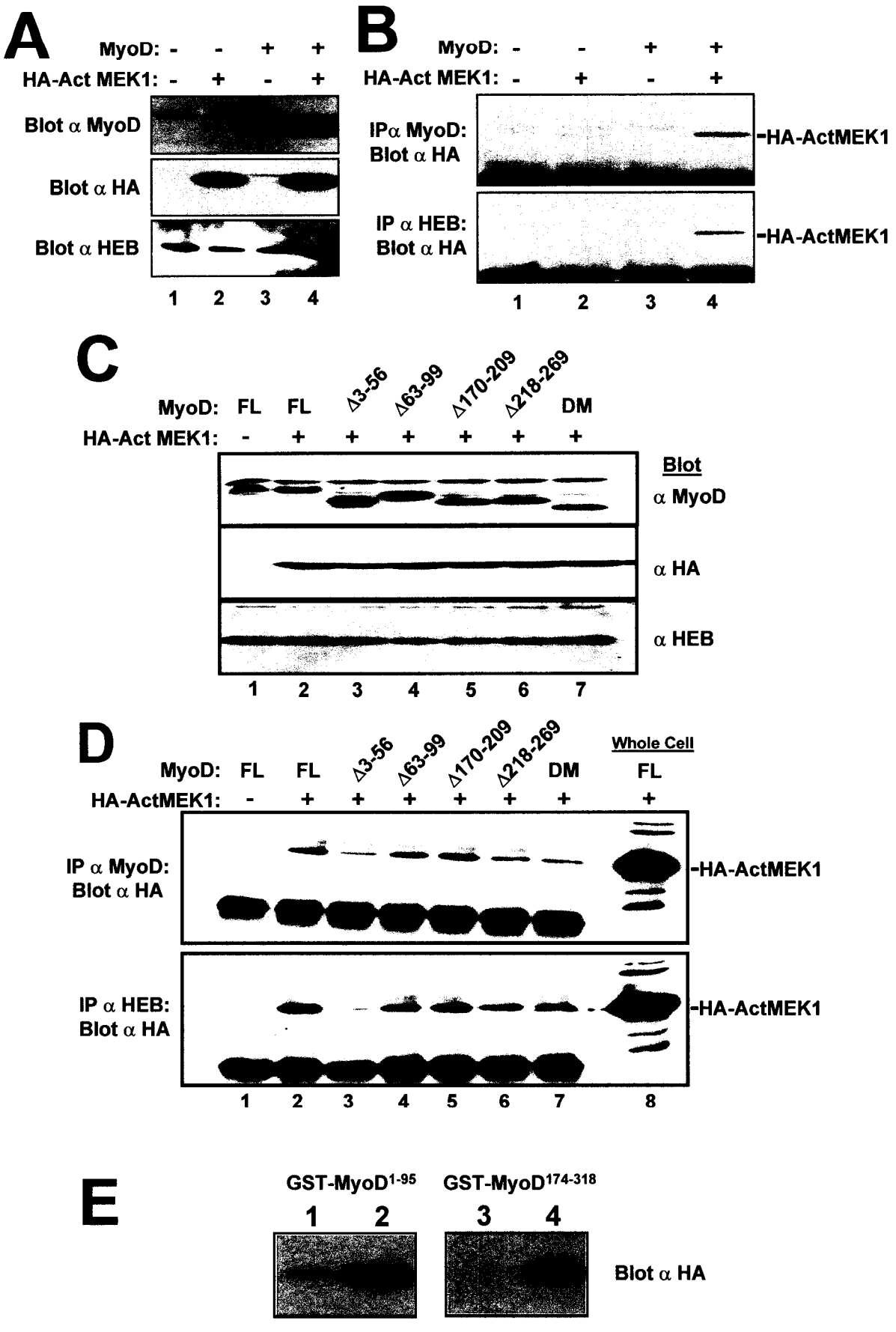
(A) Immunoblot of 10T1/2 cells transfected with MyoD and/or HA-tagged activated MEK1 showing similar levels of expression of the transfected proteins without any changes to the endogenous levels of HEB. Transfected cells were harvested 48 hours after in differentiation medium. Immunoblots were generated using 25 μg of protein extract per lane. Blots were probed as indicated.

(B) Coimunoprecipitation of HA-tagged activated MEK1 only when MyoD is present in the extracts. The extracts shown in (A) were immunoprecipitated with either anti-MyoD (top panel) or anti-HEB (lower panel) and blotted for anti-HA. In both cases, detection of MEK was only observed when MyoD was included in the transfection. This suggests that a dimer of MyoD and HEB may represent a target of MEK1. Immunoprecipitations were done as detailed in the Materials and Methods section.

(C) Cotransfection of HA-tagged MEK1 and the deletion mutants of MyoD shows equivalent levels of expression of transfected proteins and no change in HEB levels. C3H10T1/2 fibroblasts were harvested and extracted after 48 hours in differentiation medium. Immunoblots were generated using 25 μg of protein extract per lane and probed as indicated.

(D) Co-immunoprecipitation of extracts shown in (C) reveal that the N-terminal portion of MyoD is required for the formation of a complex of MyoD/HEB and MEK1

(E) GST pull-downs of extracts from MyoD/HA-tagged activated MEK1 transfected cells reveals the interaction is specific for the N-terminal portion of MyoD. The left lane in each panel represents the pull-down sample and the right lane represents 10% of the total extract used for the pull-down assay.


A
MyoD: - - + +
HA-Act MEK1: - + - +
Blot α MyoD
Blot α HA
Blot α HEB
1 2 3 4
B
MyoD: - - + +
HA-Act MEK1: - + - +
IPα MyoD:
Blot α HA
-HA-ActMEK1
IP α HEB:
Blot α HA
-HA-ActMEK1
1 2 3 4
C
MyoD: FL FL Δ3-56 Δ63-99 Δ170-209 Δ218-269 DM
HA-Act MEK1: - + + + + + +
Blot
α MyoD
α HA
α HEB
1 2 3 4 5 6 7
D
MyoD: FL FL Δ3-56 Δ63-99 Δ170-209 Δ218-269 DM Whole Cell FL
HA-ActMEK1: - + + + + + + +
IP α MyoD:
Blot α HA
-HA-ActMEK1
IP α HEB:
Blot α HA
-HA-ActMEK1
1 2 3 4 5 6 7 8
E
GST-MyoD1-95
GST-MyoD174-318
1 2 3 4
Blot α HA

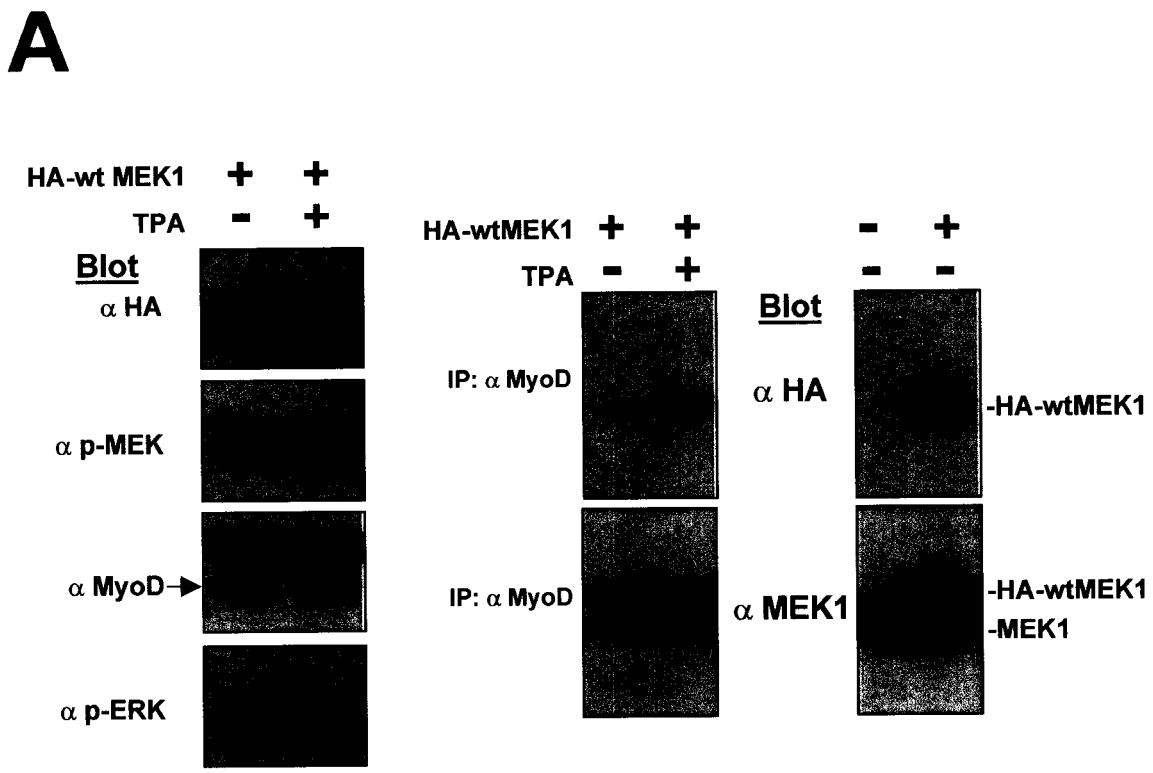
show any interaction (Figure 5.8E). Taken together, this data indicates that activated MEK1 likely represses MyoD transcriptional activity by binding directly to a MyoD/HEB complex or to a cofactor within an active transcriptional complex. Moreover, the lack of interaction of wild-type and dominant negative forms of MEK1 suggests only the activated form of MEK1 is able to interact with MyoD (data not shown; see below).

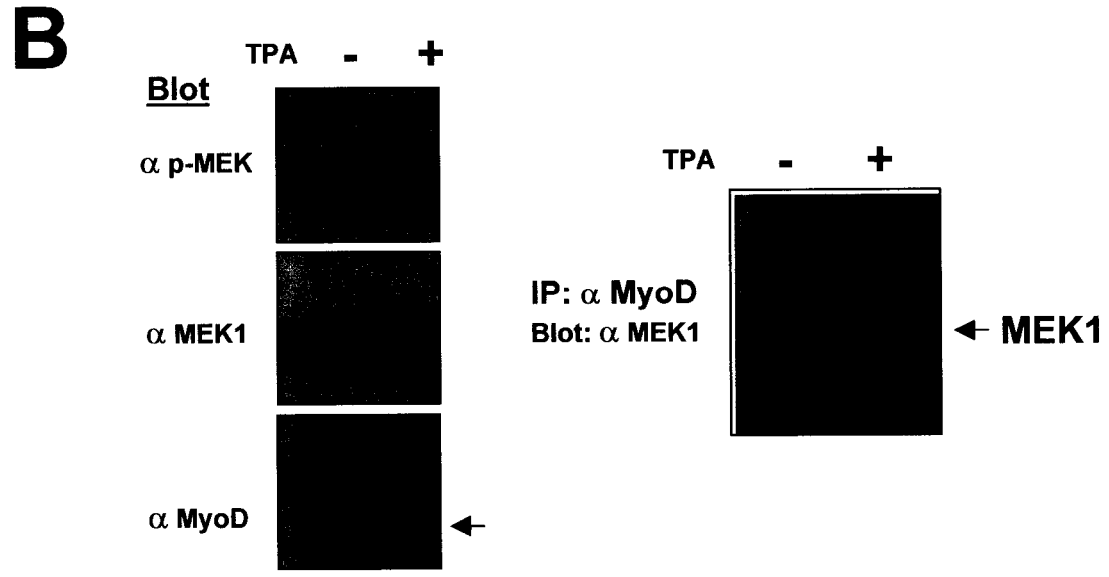
To examine the nature of this interaction in a less artificial system, C2C12 myoblast cell lines were examined by transfection of the wild-type form of MEK1 and stimulated with TPA (Figure 5.9A). It can be seen that C2C12 myoblasts do not show any changes in HA-tagged wt MEK1 expression or changes in MyoD expression after stimulation with TPA. Stimulation of cells with TPA leads to increased levels of both phosphoMEKs and phosphoERKs (Figure 5.9A; p-MEK and p-ERK blots). Immunoprecipitation of these extracts with the anti-MyoD antibody results in the detection of increased levels of HA-tagged wt MEK1 after stimulation, further supporting the requirement for MEK1 to be activated for binding to occur. Immunoblotting for MEK1 shows an increase in a doublet band of interacting proteins which represents the HA-tagged and endogenous versions of MEK1, as shown by the accompanying immunoblots (Figure 5.9A; right panels). Importantly, stimulation of untransfected, proliferating C2C12 myoblasts promotes the interaction between MyoD and MEK1 (Figure 5.9B). While immunoblots show the increases in the phosphorylation status of MEK1/2 without changes in the levels of either MEK1 or MyoD (Figure 5.9B, left panels), coimmunoprecipitation with MyoD shows increased presence of MEK1 (Figure 5.9B, right panels).

Figure 5.9: Endogenous MyoD/MEK interaction.

(A) C2C12 myoblasts were transfected with HA-tagged wt MEK1 and maintained for 24 hours in growth medium. Cells were stimulated for 5 minutes with TPA (200 ng/ml) and harvested 30 minutes post-stimulation. Immunoblotting shows increases in MEK1 and ERK1/2 activation after stimulation (compare – and + in p-MEK and p-ERK blots). Levels of MyoD and the HA-tagged wt MEK1 are unchanged irrespective of stimulation (HA and MyoD blots). Coimmunoprecipitation of reveals that both the HA-tagged MEK1 (upper band) and endogenous MEK are readily detected by immunoblotting after immunoprecipitation of endogenous MyoD. TPA treatment resulted in a dramatic increase in the amount of transfected wild-type MEK1 and endogenous MEK associated with MyoD. Immunoblots (right panels) confirm the identity of the upper and lower bands as HA-MEK1 and endogenous MEK, respectively. Immunoblots were generated using 25 μg of protein extract and probed as indicated.

(B) Coimunoprecipitation of MyoD and MEK1 from proliferating C2C12 cells after TPA stimulation. Immunoblotting revealed that TPA stimulation leads to an increase in MEK1 activation, as detected by phopho-MEK1/2 antibody, without changes in MEK or MyoD protein levels. Immunoprecipitation of endogenous MyoD resulted in a significant increase in the amount of endogenous MEK1 associated with endogenous MyoD after TPA treatment. Proliferating C2C12 cells were stimulated for 5 minutes with TPA, harvested 30 minutes post-stimulation and immunoblots were generated using 25 μg of extract and probed as indicated.


A
HA-wt MEK1 + +
TPA - +
Blot
α HA
α p-MEK
α MyoD
α p-ERK
HA-wtMEK1 + +
TPA - +
IP: α MyoD
IP: α MyoD
- +
- -
Blot
α HA
-HA-wtMEK1
α MEK1
-HA-wtMEK1
-MEK1


B
TPA - +
Blot
α p-MEK
α MEK1
α MyoD
TPA - +
IP: α MyoD
Blot: α MEK1
MEK1

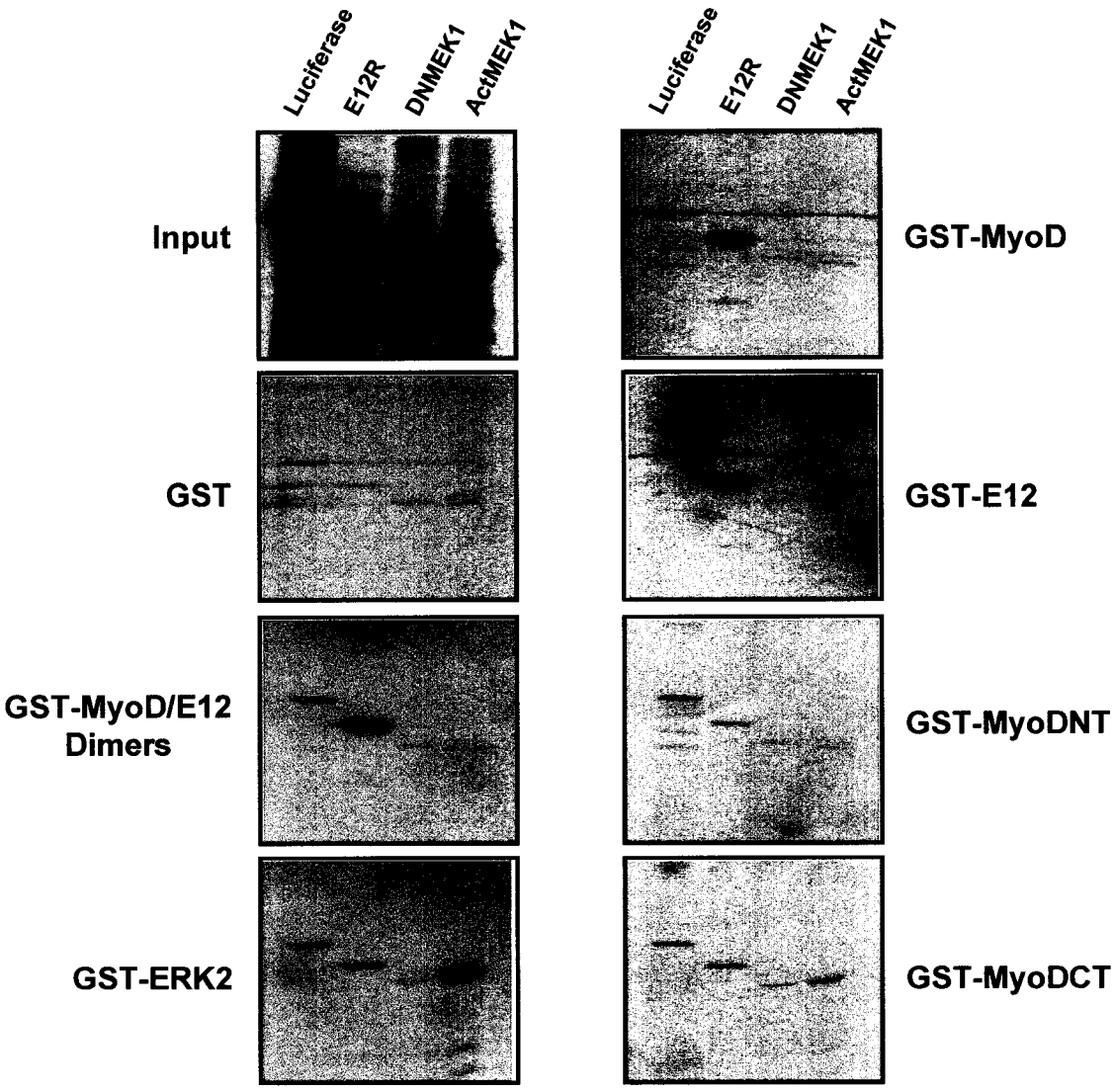
To address whether MyoD interacts directly with activated MEK1, *in vitro* translation/GST-pull-down experiments were performed (Figure 5.10). It can be seen that while full-length MyoD, full-length E12 and MyoD/E12 dimers specifically interact with a truncated version of E12 (E12R lanes), these molecules do not directly interact with activated MEK1 (Figure 4.10). Moreover, N- and C-terminal MyoD fusions do not interact with any of the *in vitro* translated products. GST-ERK2 moderately interacts with the activated version of MEK1, however, this interaction is relatively weak as levels are barely above that seen with the control, luciferase (Figure 5.10).

Together, the data shows that activated MEK1 does not affect DNA-binding or dimerization of the MRFs. Indeed, the inability of MEK1 to repress VP16-63bHLH demonstrates that the N-terminal transactivation domain represents the domain of regulation. Moreover, MEK1 binds a complex containing MyoD and HEB and this interaction requires the presence of the N-terminal TAD. However, the interaction appears to require an additional, as yet identified cofactor.

5.3 DISCUSSION

In previous chapters, data was presented showing that the MAPK signaling intermediate MEK1 regulated the transcriptional activation of MyoD. The data presented in this chapter shows a mechanism by which MEK1 regulates the activity of MyoD without affecting the stability, DNA binding or subcellular localization. While the data using the Gal4DBD fusions suggested that the N-terminal region of MyoD was unaffected by the presence of activated MEK1, data presented later in the chapter clearly demonstrate that the N-terminus is required for regulation of the full-length molecule. It should be noted that the transcriptional activity observed with the C-terminal regions

Figure 5.10: MEK/MyoD interaction is indirect and likely requires a cofactor. GST-pulldowns of *in vitro* translated proteins indicates that the interaction between MyoD and activated MEK1 is indirect and likely requires the presence of an unidenitifed cofactor. GST-MyoD and GST-E12 were able to interact with the control E12R (an N-terminal trucated form of E12). This interaction requires the bHLH domain as neither the N- or C-terminal regions of MyoD demonstrate interaction. GST-ERK2 modestly interacted with activated MEK1 whereas none of the fusions of MyoD or E12 were able to interact with dominant negative or activated forms of MEK1. The input panel shows the efficiency of *in vitro* translation and labeling.


Luciferase
E12R
DNMEK1
ActMEK1
Luciferase
E12R
DNMEK1
ActMEK1
Input
GST-MyoD
GST
GST-E12
GST-MyoD/E12
Dimers
GST-MyoDNT
GST-ERK2
GST-MyoDCT

differs from previous reports (Weintraub *et al.*, 1991) and may reflect a difference in the nature of the constructs. The lack of interaction between MyoD and MEK1 when the amino-terminal domain is absent or, via the direct *in vitro* translation indicates that the interaction is indirect and may require the expression of a myoblast specific gene, absent from cells expressing the TAD deletion mutant. Alternatively, downstream kinases of the ERKs, such as Rsk, may play a role.

MyoD recruits factors important for chromatin remodeling (Gerber *et al.*, 1997; Puri *et al.*, 1997a; Puri *et al.*, 1997b; Sartorelli *et al.*, 1997) and can directly interact with elements of the basal transcriptional machinery (Heller and Bengal, 1998). Also, MyoD requires acetylation of lysine residues close to the basic DNA-binding domain for transcriptional activation (Sartorelli *et al.*, 1999; Tintignac *et al.*, 2000). It is possible that the active form of MEK1 disrupts these interactions by phosphorylating some component of the MyoD transcriptional complex. Indeed, the N-terminus of MyoD is required for interaction with p300 (Sartorelli *et al.*, 1999). In addition, such a role for MEK1 would appear not to involve the ERKs because other activated mutant forms of MEK1 remain cytoplasmic but activate ERK1/2 to the same level as MEK1 ΔN3 S218E/S222D used in this study. However, it is also possible that ERK1/2 was not observed to associate with the MyoD-MEK1 complex due to dissociation of activated ERK from the complex (data not shown).

The experiments presented in this chapter demonstrate that MEK1-mediated repression of MyoD function was targeted to the N-terminal TAD and not to the heterodimerization, DNA binding or chromatin-remodeling domains. Truncated mutants of MyoD lacking the C-terminal portion were repressed upon coexpression with activated

MEK1. By contrast, MEK1 did not repress muscle-specific gene expression when the VP16 acidic TAD was substituted for the N-terminal TAD of MyoD. Although DNA binding and changes in dimerization status have been shown as mechanisms for MRF regulation, the fact that activated MEK1 was unable to repress the VP16 TAD when fused to the MyoD bHLH indicates these regulatory mechanisms were not involved. This notion is strengthened by the fact that myogenin expression, which requires chromatin-remodeling function, was induced by VP16-63bHLH and was unaffected by the presence of activated MEK1.

Whether MyoD impacts on the downstream kinase activity of MEK1 remains unclear. Reports examining the role of the MEK1 upstream activator Mos demonstrate that activated Mos stimulates myogenesis (Lenormand *et al.*, 1997; Pelpel *et al.*, 2000). In addition, MyoD is capable of inhibiting Mos activation of MEK1 (Solhonne *et al.*, 1999). This alternate function of MyoD, beyond that of activating gene expression, raises the possibility that MyoD protein may regulate MEK1 downstream activity. A coordinated regulatory circuit such as this delineates a model of how growth factors can utilize the MAPK pathway to modulate the myogenic program without a concomitant loss of myoblast identity. The interaction of activated MEK1 with the MyoD/HEB transcriptional complex represents a novel mechanism of how signaling pathways can regulate myogenesis.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

The purpose of the research presented in the preceding chapters was to further understand the role of the MRFs in determining the myogenic lineage and to gain insight into the underlying molecular mechanism by which peptide growth factors act to repress terminal differentiation. The data presented provides clear evidence that skeletal muscle termination differentiation does not require *MyoD* or *Myf5* while maintenance of the myogenic lineage does require expression of a functional MyoD and/or Myf5 molecule. The cell lines generated for these analyses also demonstrate that there is not an underlying, non-MRF genetic program that permits maintenance of myogenic identity.

Examination of the molecular mechanism of growth factor-mediated regulation of myogenesis revealed a novel mechanism by which activated MEK1 is capable of directly interacting with a transcription complex containing both MyoD and its dimerization partner HEB. This interaction requires the N-terminal transactivation domain of MyoD and likely requires a cofactor. Moreover, this research also found a novel, and potentially critical, regulatory mechanism for myogenic differentiation by finding that the MRF dimerization partner HEB appears to be targeted for degradation or tissue-specific downregulation of gene expression during the G1-S to G2-M transition periods of the myoblast cell cycle.

To understand the role of the MRFs in determining the myogenic lineage and initiating terminal differentiation, transfection studies were performed using fibroblast cell lines lacking functional copies of *MyoD* and *Myf5* genes. Initial experiments

demonstrated that myogenin and MRF4 are competent for initiating terminal differentiation, indicating MyoD and Myf5 are dispensable for activating the myogenic program. It remains to be seen whether proliferating DKO fibroblasts expressing myogenin and MRF4 are specified to the myogenic lineage or, if these two MRFs merely support terminal differentiation in the absence of MyoD and Myf5 expression. Indeed, analyses using an inducible form of MyoD demonstrated that maintenance of the myogenic lineage requires the continued expression of a functional MyoD molecule, supporting the notion that MyoD and Myf5 are required for lineage maintenance. This data also shows that an underlying, non-MRF myogenic subprogram does not exist.

The lack of functional copies of *MyoD* and *Myf5* genes in the DKO fibroblast cell lines generated for this work represent a unique opportunity with which to study the specific genes activated by MyoD and Myf5. Studies using gene chip technology to determine the distinct gene targets activated by MyoD and Myf5 are currently underway (Ishibashi, J. and Rudnicki, M.A.). Furthermore, since lineage acquisition is reversible due to the inability of the endogenous myogenic program to be activated, use of a dual-selection gene-trap system (Medico *et al.*, 2001) should permit the identification of MyoD target genes. Identification of myoblast-specific genes would represent an important advance toward understanding how cells maintain lineage during the proliferative phase of the myogenic program and possibly, lead to the identification of important cooperative factors that are vital to myogenic specification.

To gain understanding of the underlying molecular mechanisms involved with the ability of growth factors to repress the myogenic program, experiments were carried out assessing the effect of the MAPK pathway intermediate MEK1 on MRF-mediated

transcriptional activity. Transient transfection studies clearly demonstrate that expression of an activated form of MEK1 represses the ability of the MRFs to activate transcription and initiate the myogenic program. Importantly, the regulation is not mediated by changes in MRF stability, subcellular localization or direct phosphorylation by the ERKs or JNKs. An important aspect of the regulation is the localization of activated MEK1 to nucleus of myoblasts. As observed with other cell types (Tolwinski *et al.*, 1999), MAPK pathway activation leads to the localization of endogenous MEK1 to the nuclear compartment of myoblasts and MEK1/2 shows an increase in activation during the G2-M transition of the cell cycle when MyoD protein levels are increasing. Since inhibition of MAPK signaling does not force differentiation of proliferating myoblasts, there are likely alternative pathways involved with inhibition of myogenesis. By contrast, inhibition of MEK1 signaling advances differentiation and reduces the ability of myoblasts to proliferate under low-mitogen conditions indicating a link with MAPK signaling, cell cycle exit and MyoD-mediated activation of differentiation. Interestingly, prolonged exposure of differentiating myotubes to the MEK1 inhibitor, U0126, clearly points to a role for MAPK signaling during fusion. This effect may be due to a decrease in ERK activity, as translocation of MEK1 to the nucleus does not occur in cells that are differentiating or, maybe a consequence of reduced MEK5 activity.

Experiments focused on understanding the nature of MAPK regulation clearly indicate that an interaction between activated MEK1 and the MyoD transcriptional complex occurs. Of particular importance is the fact that the N-terminal transactivation domain (TAD) of MyoD is required for this interaction and the complex likely contains an additional coactivator. This coactivator may be specific for skeletal muscle cells as

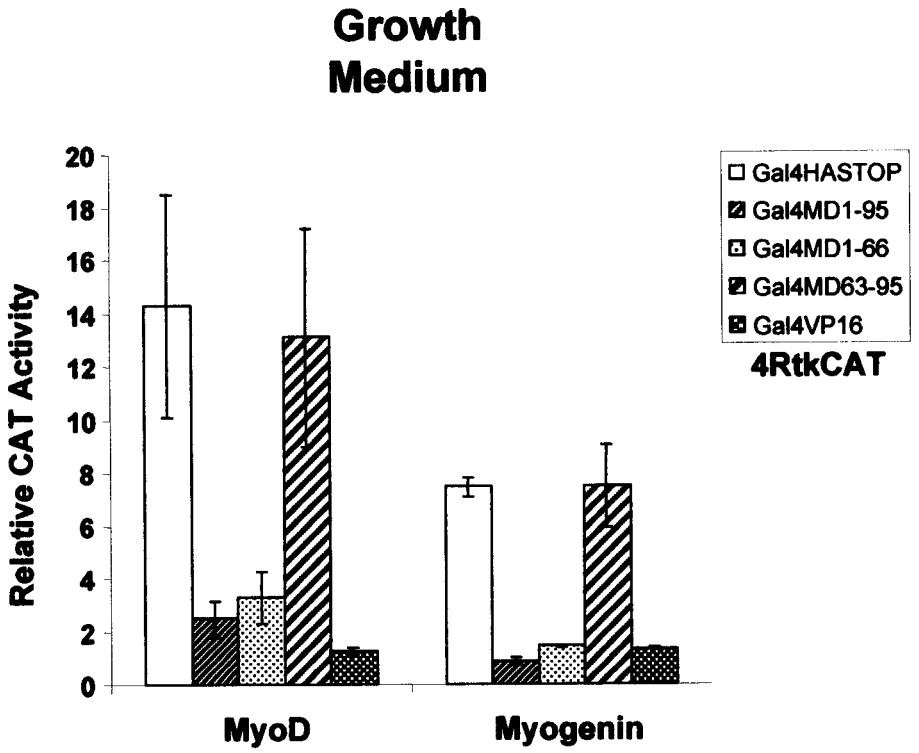
preliminary data examining the interaction shows that a GST-MyoDNT/MEK1 interaction is only detected using extracts from fibroblasts coexpressing MyoD and is not detected if activated MEK1 is transfected alone (not shown). Further evidence for the involvement of a muscle-specific transcriptional coactivator can be seen by the fact that expression of the amino-terminal transactivation domain of MyoD fused to the Gal4DBD dramatically interferes with the ability of full-length MyoD and myogenin molecules to activate transcription (Figure 6.1). Under both proliferative (Figure 6.1A) and low-mitogen (Figure 6.2B) conditions, the ability of both MyoD and myogenin to activate a skeletal muscle-specific reporter vector is severely compromised when the amino-terminal transactivation domain of MyoD is coexpressed. This suggests a soluble factor is competed away by the presence of the MyoD-TAD, as the chromatin-remodeling domain does not alter transcriptional activity (Figure 6.1).

The specificity of this effect is seen by coexpression of the same fusions of MyoD with other transcription factors (Figure 6.2). Neither the Ets-domain transcription factor Pea3 or the MADS-box containing MEF2C are affected in their ability to activate specific reporter vectors when the N-terminal TAD of MyoD is coexpressed. This lack of effect is observed under both proliferative (Figure 6.2A) and low-mitogen conditions (Figure 6.2B). It should be noted that the repressive effect of the Gal4-VP16 fusion on MyoD and myogenin (Figure 6.1) is also observed with both Pea3 and MEF2C (not shown). Since the VP16 TAD is capable of interacting with elements of the basal transcriptional machinery (Sadowski *et al.*, 1988; Uesugi *et al.*, 1997), the effect of this protein domain likely occurs with most transcription factors. However, the inability of the N-terminal TAD of MyoD to affect Pea3 and MEF2C strengthens the involvement of

Figure 6.1: N-terminal TAD of MyoD inhibits MRF-mediated transcription.

To address the specificity of MRF-mediated gene transcription and the potential that a skeletal muscle-specific cofactor exists, 10T1/2 fibroblasts were transfected with plasmids encoding full-length MyoD or myogenin, the N-terminal domains of MyoD fused to the Gal4-DBD or the Gal4-VP16 fusion and the 4RtkCAT. Cells were harvested and CAT assays performed after 48 hours in growth (Panel A) or low-mitogen (Panel B) conditions. Under both conditions, it can be seen that coexpression of Gal4-DBD fusions containing the N-terminal TAD of MyoD (amino acids 3-56) dramatically reduced the ability of both MyoD and myogenin to activate muscle-specific transcription. In all cases, the Gal4-VP16 fusion interferes with MRF-mediated transcription. The data shown is one representative experiment. Bars represent the means and error bars representing the standard error of the mean (+/- SEM; n=3).

A


Growth Medium
Relative CAT Activity
20
18
16
14
12
10
8
6
4
2
0
MyoD
Myogenin
Gal4HASTOP
Gal4MD1-95
Gal4MD1-66
Gal4MD63-95
Gal4VP16
4RtkCAT

B

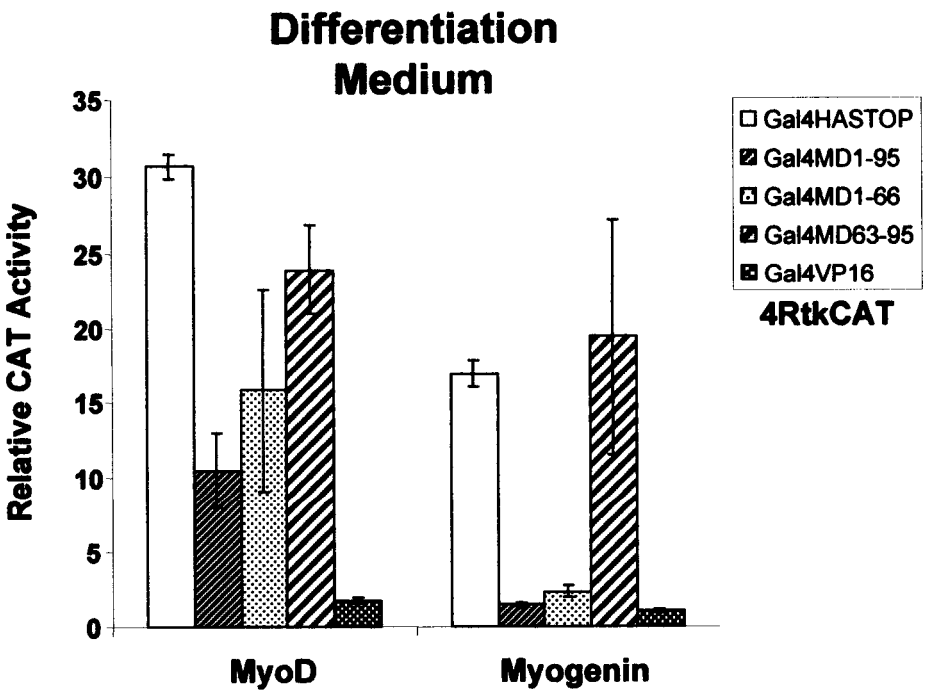
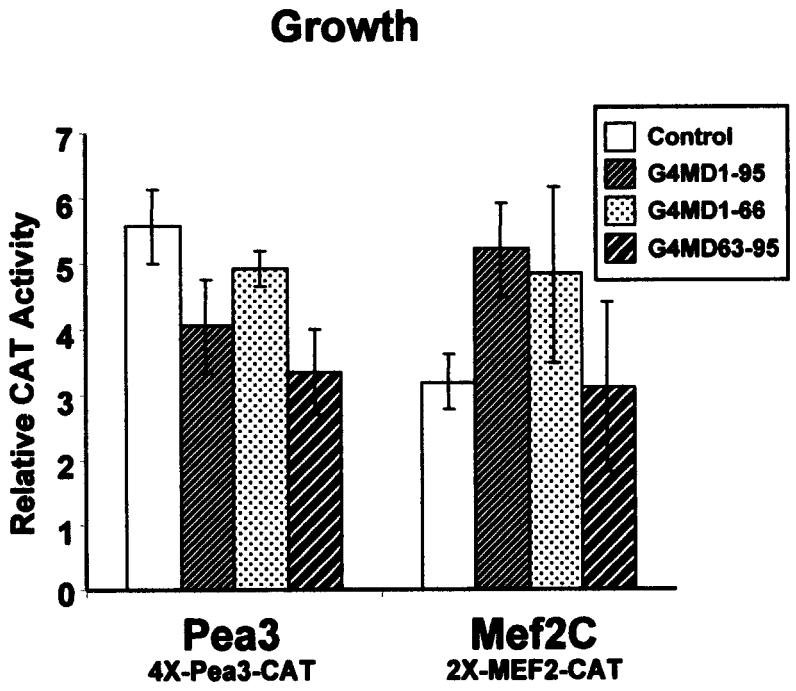

Differentiation Medium
Relative CAT Activity
35
30
25
20
15
10
5
0
MyoD
Myogenin
Gal4HASTOP
Gal4MD1-95
Gal4MD1-66
Gal4MD63-95
Gal4VP16
4RtkCAT

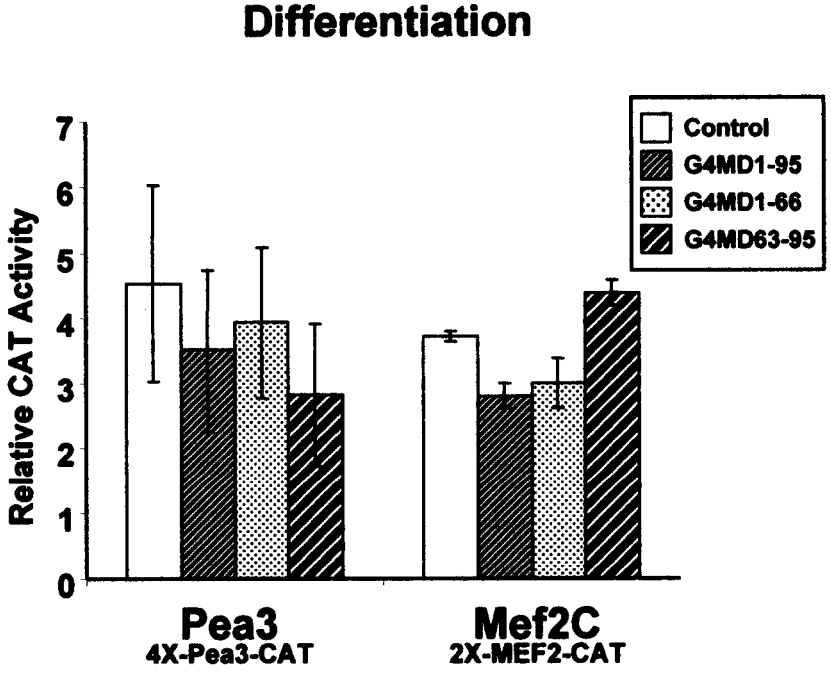
Figure 6.2: N-terminal TAD of MyoD does not inhibit other transcription factors

To assess whether the N-terminal TAD of MyoD affects transcriptional activity non-specifically, the transcriptional activity of non-related transcription factors was examined. Fibroblasts were transfected as indicated, harvested after 48 hours under growth (Panel A) or low-mitogen (Panel B) conditions and CAT assays were performed. Transcriptional activation by Pea3 was determined using a 4X-Pea3-CAT while MEF2C activity was measured using a 2X-MEF2-CAT reporter vector. It can be seen that coexpression of the N-terminal MyoD Gal4-DBD fusions do not affect the transcriptional activity of either Pea3 or MEF2C. This indicates the repressive effect of the N-terminal TAD of MyoD is specific for MRF-mediated gene activation. It should be noted that the Gal4-VP16 fusion efficiently reduces both Pea3 and MEF2C transcriptional activity (data not shown). Shown is one representative experiment. Bars represent the means and error bars the standard error of the mean (+/- SEM; n=3).

A


Growth
Control
G4MD1-95
G4MD1-66
G4MD63-95
Relative CAT Activity
0
1
2
3
4
5
6
7
Pea3
4X-Pea3-CAT
Mef2C
2X-MEF2-CAT

B


Differentiation
Control
G4MD1-95
G4MD1-66
G4MD63-95
Relative CAT Activity
0
1
2
3
4
5
6
7
Pea3
4X-Pea3-CAT
Mef2C
2X-MEF2-CAT

a myoblast specific coactivator. The lack of a muscle-specific coactivator may explain the inability of activated MEK1 to repress Gal4-MyoDNT fusions. Similarly, multiple levels of regulation likely exist as all domains of MyoD appear to be affected by activated MEK1, as seen using both Gal4-fusions and MyoD deletion mutants. Furthermore, the ability of the deletion mutant of MyoD lacking the N-terminal TAD to convert fibroblasts to the myogenic lineage indicates the multiple mechanisms involved in MyoD-mediated gene activation and myogenesis. It is clear that experiments to identify molecules interacting with MRF transcriptional complex are of great importance. Moreover, characterization of whether MEK1 interacts with the other MRFs will be of interest to determine specificity.

Analyses focused on understanding the role of MEK1 during the cell cycle revealed a surprising regulatory mechanism revolving around the stability of the MRF dimerization partner HEB. Since MEK1 nuclear translocation occurs at the G2-M boundary, extracts from synchronized C2C12 cells were analyzed for an interaction between activated MEK1 and MyoD. Coimmunoprecipitation experiments were unable to detect an interaction at the G2-M boundary of the cell cycle (not shown). This lack of interaction appears to be due to the fact that immunoblotting of these extracts showed that the MRF dimer partner HEB is not detectable during the G1-S to G2-M phases of the cell cycle. This suggests that terminal differentiation is regulated by the specific repression of HEB expression or targeted protein degradation of a dimerization partner of MyoD. The significance of this is now being revealed by studies continuing in the laboratory (Parker, M. and Rudnicki, M.A.). Indeed, preliminary data has shown that under proliferative conditions the alpha isoform of HEB is capable of synergystically activating MyoD-

mediated transcription. Under differentiation conditions, HEB-alpha switches preferences to myogenin. This data strongly implicates HEB as the proliferative dimer partner for MyoD and suggests that the initial stages of myogenesis rely on this dimer pair to activate the myogenic program. It is of great importance to establish how HEB expression is regulated during the final cell cycle phase at the time that a myoblast enters the differentiation phase.

The results presented here, in association with reports demonstrating p38 MAPK activation stimulates myogenesis (Puri *et al.*, 2000; Wu *et al.*, 2000; Zetser *et al.*, 1999), indicate that the coordinated activity of distinct MAPK pathways likely regulate protein-protein interactions important for MRF-mediated gene expression. The necessity of MyoD for lineage specification and the demonstration that MEK1 can interact with a transcriptional complex containing MyoD suggests the possibility that modulation of specific protein complexes under different conditions may alter the spectrum of genes activated by the MRFs (Figure 6.3). Since the ERKs are not directly involved, future studies should address the potential that ERK-activated kinases, such as the Rsks, may target the MRF transcriptional complex.

The results presented within this thesis describe: i) the creation of cell lines that are reversible in their acquisition of the myogenic lineage and will be invaluable for studying the activities of specific MRFs; ii) the description of a novel level of regulation by the interaction of MEK1 with a transcriptional complex of MyoD and; iii) the first description of a regulatory system controlling myogenesis by the cell cycle-specific regulation of the MRF dimerization partner HEB. Clearly, future experiments characterizing the individual components of MyoD transcriptional complexes will

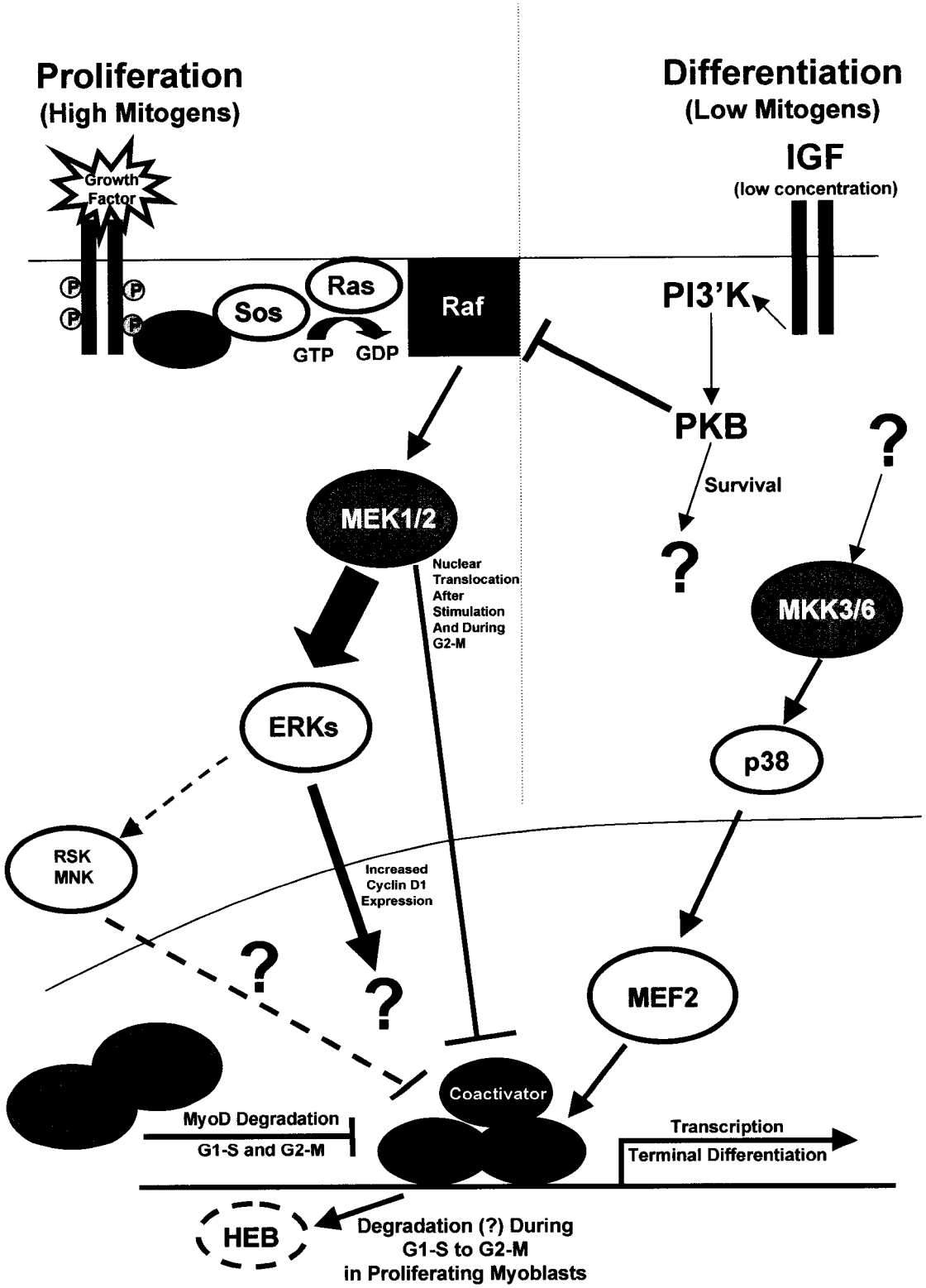
provide an important step forward in understanding not only transcriptional activation but the nature of how individual tissue lineages arise and are maintained.

Figure 6.3: Model of signal transduction control of myogenesis.

Shown is a model of how the MAPK pathways may coordinately regulate myoblast proliferation and differentiation while permitting the cells to maintain their myogenic identity.

Proliferative conditions: Growth factors bind to their cognate receptors leading to activation of the ERKs through ras, raf and MEK1/2. Most MEK1/2 activity is directed toward activating ERK1/2 while some activated MEK1 translocates to the nucleus. Nuclear MEK represses myogenic gene activation by binding the transcriptional complex containing MyoD/HEB heterodimers and an as yet identified coactivator. The interaction of MEK with a MyoD transcriptional complex does not alter the subcellular localization or stability of MyoD. Typically, high ERK activity leads to increased cyclin D1 expression, inhibiting myogenesis by direct binding to MyoD. It is currently unclear whether other ERK-activated kinases have a negative impact on MRF-mediated gene expression. During the cell cycle, distinct cyclin/cdk1/2 complexes target MyoD for degradation and, by an as yet unknown mechanism, HEB is not present during the G1-S to G2-M transition phases of the cell cycle. The absence of HEB prevents MyoD-mediated gene activity due to the lack of a dimerization partner.

Differentiation conditions: Removal of mitogens reduces MEK activation/nuclear localization thereby, downregulating ERK activation which leads to decreased cyclin D1 levels. Stimulation of cells with differentiation inducing factors, such as low concentrations of IGF, activates PKB which, in turn, suppresses MEK activation via raf inhibition. Increases in p38 activation leads to activation of MEF2-mediated gene expression, further potentiating terminal differentiation.


Proliferation
(High Mitogens)
Differentiation
(Low Mitogens)
IGF
(low concentration)
Growth Factor
Sos
Ras
Raf
GTP
GDP
PI3'K
PKB
Survival
?
MEK1/2
Nuclear Translocation After Stimulation And During G2-M
MKK3/6
ERKs
p38
RSK
MNK
Increased Cyclin D1 Expression
?
?
MEF2
Coactivator
MyoD Degradation
G1-S and G2-M
Transcription
Terminal Differentiation
HEB
Degradation (?) During
G1-S to G2-M
in Proliferating Myoblasts

CHAPTER 7

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