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INHIBITION OF SKELETAL MYOGENESIS BY ACTIVATED RAF-KINASE

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ABSTRACT

The mobility of the human body is dependent upon the contractile function of skeletal muscle. Proper formation of skeletal muscle is a complex process involving specification of mesodermal progenitors to a muscle lineage, proliferation of myoblasts, and subsequent differentiation of the myoblasts into myofibers that produce the proteins required for muscle contraction. Initiation of intracellular signaling cascades by growth factor binding to cell surface receptors precisely controls myogenesis. Many factors are capable of disrupting muscle formation by interfering with various components involved in the process. Ras is a membrane localized GTPase activated by many growth factors that is capable of inducing multiple signaling pathways, one of which is the conventional Raf/MEK/MAPK pathway. Constitutively activated forms of Raf-kinase induce morphological transformation and are inhibitory to terminal differentiation of skeletal muscle cells in vitro. The activation of the transcription factor activator protein-1 (AP-1) by this kinase appears to be responsible for the cellular changes in phenotype. However, the mechanism behind the block to differentiation is independent of this phenomenon and remains unidentified.

Raf-kinase exerts its effects by altering the expression of genes that may positively or negatively affect muscle fiber formation. Several putative Raf-regulated genes were identified in a subtractive library screen of Raf-transduced myoblasts. One of these genes was slightly downregulated in Raf-transformed avian myoblasts and was found to be highly homologous to a human Tax Responsive Element Binding Protein 107 (TaxREB107). We pursued the

possibility that chick TaxREB107 (cTaxREB107) may be involved in transcriptional regulation during myogenesis. Our findings suggest a positive role for cTaxREB107 in the regulation of muscle gene expression during development of skeletal muscle. Therefore, expression of cTaxREB107 may be impeded by Raf-kinase in order to negatively affect myogenesis.

To further extend our understanding of the block to myogenesis that is imposed by activated Raf-kinase, a stable myogenic cell line expressing an estrogen receptor-Raf chimeric protein was created. Induction of high levels of Raf-activity was found to prevent fiber formation. Interestingly, conditioned media from the Raf-inducible cell line inhibited fiber formation in the parental cell line, which suggests the possibility that a secreted factor is responsible for the block to differentiation of skeletal muscle cells. Gene expression and activity of *transforming growth factor β_1 ($TGF\beta_1$)*, which is a well-known inhibitor of the myogenic program, was found to be elevated in cells expressing high levels of activated Raf-kinase. However, addition of a $TGF\beta_1$ inhibitor did not restore muscle fiber formation in these cells. These results suggest that although *$TGF\beta_1$* expression and activity is increased, the induction of this growth factor is not solely responsible for the block to myogenesis that is imposed by activated Raf-kinase.

Although cellular secretion of $TGF\beta_1$ is not necessarily the cause of the Raf-imposed block to muscle formation, a TGFβ-like factor is probably responsible for these effects. Treatment of myoblasts with exogenous TGFβ has been shown to suppress the accumulation of MEF2 to the nucleus. MEF2

retention in the cytoplasm also occurs in muscle cells overexpressing activated Raf-kinase, thereby providing a link between TGFβ-induced and Raf-induced myogenic inhibition. Therefore, we chose to study the interaction of the TGFβ and Raf-kinase pathways during myogenesis. Our results indicate that Raf-kinase and TGFβ similarly induce transcriptional activity of activator protein (AP-1). Fra-2 and c-Jun were found to be the primary AP-1 DNA binding components in Raf-expressing myoblasts as well as those treated with TGFβ. However, Western blot analysis of both TGFβ-treated and Raf-expressing myoblasts revealed that Fra-1 protein levels were elevated. AP-1 tether proteins were used in order to analyze the effects of specific subunit composition of AP-1 on myogenesis. c-Jun~Fra-1 and c-Jun~Fra-2 both suppressed muscle-specific reporter gene activity and were inhibitory to myofiber formation. Furthermore, c-Jun~Fra~1 was found to directly affect MEF2 function, while c-Jun~Fra-2 was shown to be inhibitory to MRF activity. This data provides evidence for specificity of AP-1 components in the regulation of subsets of genes during skeletal myogenesis.

In conclusion, the data provided here suggests that Raf-kinase controls a complex pattern of gene expression. While the exact mechanism behind the Raf-imposed block to myogenesis has not been completely elucidated, the results of this study indicate that Raf regulates expression of cTaxREB107, members of the TGFβ superfamily and specific AP-1 subunits.

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Chapter 1

LITERATURE REVIEW

I. Skeletal Myogenesis:

The formation of skeletal muscle, also known as skeletal myogenesis, is a complex developmental process that occurs in multiple, well-delineated steps. The vast majority of skeletal muscle develops from somitic cells during embryogenesis (Brand-Saberi and Christ, 1999). Mesodermal progenitors in the dermomyotome are first specified to the myogenic lineage. The committed myoblasts then proliferate to form a large pool of cells and migrate to their proper sites (Pourquie et al, 1995). When the cells receive environmental cues such as growth factor withdrawal to exit the cell cycle, they terminally differentiate into multinucleate myofibers (Lassar et al, 1994).

Muscle fibers are the basic units of skeletal muscle that allow the tissue to perform the physiological function of contraction to aid in movement of an organism. Differentiated muscle cells express proteins that are necessary for the contractile apparatus such as actin, myosin, and troponin. Growth factors are important regulators of skeletal muscle development and regeneration because of their ability to activate various intracellular signaling cascades that relay information to the nucleus. Trancriptional activators are the endpoints of the signaling pathways and control transcription from muscle-specific genes (Lassar et al, 1994). Each participant in the growth factor axis has the ability to both negatively and positively regulate myogenesis. All are necessary components to precisely control the events leading up to proper skeletal myogenesis.

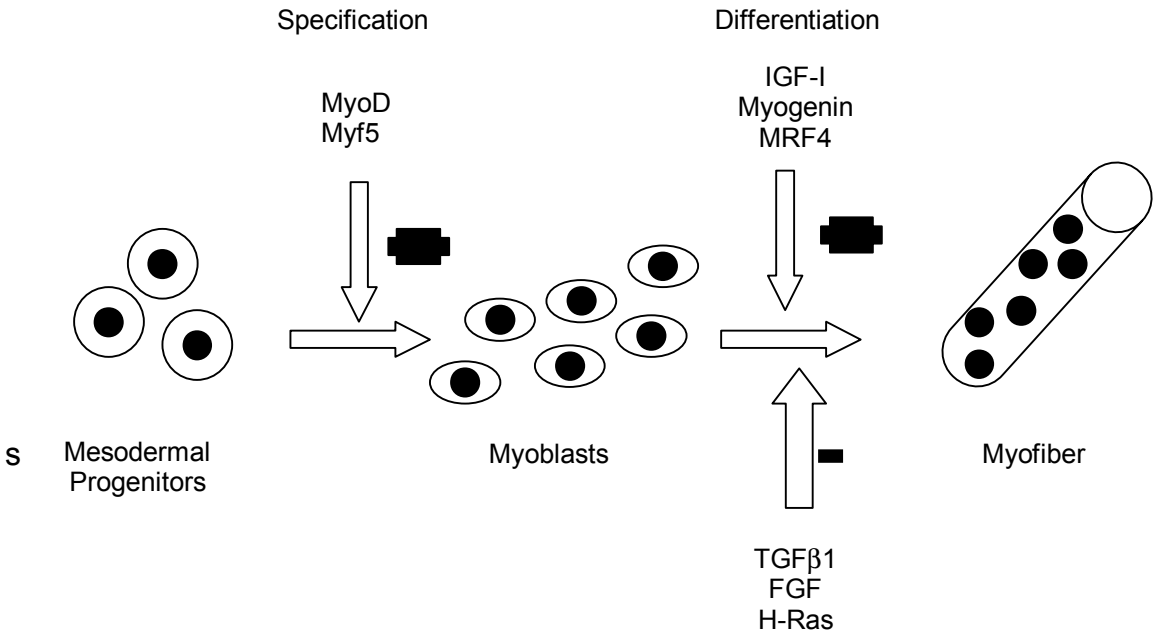

Figure 1.1. Skeletal myogenesis.

II. Transcriptional regulation of skeletal myogenesis

Myogenic Regulatory Factors:

The myogenic regulatory factors (MRFs) are a family of transcriptional activators whose expression is restricted to skeletal muscle. MyoD was the first MRF isolated in a subtractive screen and was thought to be the master regulatory gene for myogenesis on the basis that it was able to convert C3H10T1/2 fibroblasts into myoblasts (Davis et al 1987). The cloning of MyoD was followed quickly by the discovery of myogenin, Myf-5, and MRF4 (Wright et al, 1989; Braun et al, 1989a; Rhodes et al, 1989). Each of these related factors is sufficient to activate a complement of muscle-specific genes in a number of non-myogenic lineages as well as the expression of one or more of the other

three MRFs (Weintraub et al, 1989;Braun et al, 1989b). The protein domain map of each MRF contains two important motifs: a DNA-binding and dimerization motif and one or more transcriptional activation domains. Both components are necessary for the tissue-specific actions of the muscle regulatory factors.

The DNA binding and dimerization motif present in the MRFs is a highly conserved region known as the basic helix-loop-helix (bHLH) domain, which is composed of a basic region followed by two amphipathic α-helices separated by an intervening loop (Murre et al, 1989a). Many proteins capable of binding DNA contain this important domain, including E12 and E47, two alternatively spliced products of the E2A gene (Murre et al, 1989a). The necessity of the HLH domain for dimerization was demonstrated by site-directed mutagenesis of the E47 gene (Voronova et al, 1990). Upon dimerization, the HLH motif brings together two basic regions to form a composite DNA binding domain. Although the MRFs are capable of homodimerization, the affinity of the MRFs for the muscle-specific genes increases dramatically when the MRFs form heterodimers with E12 and E47 (Murre et al, 1989b; Lassar et al, 1991; Brennan and Olson, 1990). Recent evidence suggests that the expression of individual muscle genes may be controlled by heterodimerization of each MRF with specific E-proteins (Becker et al, 2001).

The basic region of each member of the bHLH protein family is capable of binding DNA at sequence-specific DNA elements in order to activate transcription. These elements, known as E-boxes, have a consensus nucleotide sequence of CANNTG and are present in the control regions of many muscle-

specific genes such as myosin light chain, the troponin I enhancer, and muscle creatine kinase (Davis et al, 1990; Braun et al 1990; Wentworth et al, 1991; Lassar et al, 1989). Although mutation of the basic region inhibits DNA binding, dimerization capacity is not affected (Brennan et al, 1991a). Specificity of the basic region of the MRFs for transcriptional activation of muscle-specific genes was demonstrated by domain swapping experiments where the basic region of the MyoD protein was replaced with the basic region of the E12 protein. The resulting chimeras dimerized with E-proteins and bound E-boxes, but did not activate transcription of muscle specific genes (Davis et al, 1990). Therefore, while the basic region is important for muscle-specific transcription, DNA binding alone is not adequate for the initiation of myogenesis. Two adjacent amino acids residues in the basic region of MyoD, Alanine 114 and Threonine 115, are sufficient for muscle-specific transcriptional activation as well as conversion of fibroblasts to myoblasts when substituted into the corresponding region of E12 (Brennan et al, 1991b; Davis et al, 1992). These residues are conserved in the MRFs, but are not present in any of the other bHLH family members. A mutant form of MyoD in which Alanine 114 was replaced with an asparagine had no effect on the efficiency of DNA binding, but dramatically decreased transcriptional activation (Bengal et al, 1994). The mechanisms of action by these muscle-specific amino acid residues are unknown, but they may be required for interaction of the MRFs with a co-factor, such as the myocyte enhancer factor 2 (MEF2). While the bHLH domains are required for transcriptional activation of

target genes, the motif alone is transcriptionally inactive and depends on other domains in order to activate transcription (Schwarz et al, 1992; Black et al, 1998).

Although the muscle regulatory factors share a high degree of homology in their bHLH domains, the amino acid sequences of their NH_2- and COOH-termini are very different (Schwarz et al, 1992). This suggests that the specific biological activities of each MRF are regulated in these divergent domains. Transcriptional activation domains (TADs) are elements in the amino and carboxy terminal regions of the MRFs that are required for muscle-specific gene activation. Without these domains, for example, myogenin is incapable of converting fibroblasts to myoblasts or activating exogenous muscle-specific genes (Schwarz et al, 1992). Specificity of individual MRF TADs was demonstrated by swapping the amino and carboxy termini of MyoD and MRF4 (Mak et al, 1996). Normally only myogenin efficiently activates the enhancer of the muscle creatine kinase, although MRF4 is capable of binding and may weakly activate transcription (Yutzey et al, 1990). However, when the transcriptional activation domains of MRF4 and MyoD were exchanged, the specificities of these factors for the muscle creatine kinase enhancer also were interchanged (Mak et al, 1996). Experiments using the yeast GAL4 binding domain fused to the activation domains of MyoD showed transcriptional activity in cells where they are normally transcriptionally inactive (Weintraub et al, 1991). This finding suggests that the TADs in muscle regulatory factors do not require a cell-specific context to exert their effects.

Because each of the MRFs is capable of initiating the entire muscle program in cultured non-muscle cells, a functional redundancy of the factors was initially thought to exist. However, each MRF has a distinct pattern of expression, which suggests that all of the factors have different functions in vivo. *Myf-5* is the first MRF expressed in the mouse embryo at embryonic day 8.0, although levels of expression decrease rapidly by embryonic day 10.5 (Ott et al, 1991). Expression of *myogenin* appears 12 hours after *Myf-5*, followed closely by *MRF-4* at embryonic day 9.0, and lastly, *MyoD* at embryonic day 10.5 (Bober et al, 1991). *MRF-4* has a transient pattern of expression, while *MyoD* and *myogenin* persist throughout development of the mouse (Sassoon et al, 1989).

Analysis of MRF expression in established cell lines, as well as gene ablation experiments, has revealed the function of each factor during the myogenic process. *MyoD* and *Myf-5* are expressed in myoblasts, but the *MyoD* knockout mouse is viable and has no defects in skeletal muscle (Rudnicki et al, 1992). Interestingly, *Myf-5* was upregulated five-fold in the *MyoD* null mice, which suggests a compensatory mechanism for the lack of *MyoD*. Although skeletal muscle formation is normal, gene targeting of *Myf-5* in mice results in perinatal death and severe rib defects (Braun et al, 1992). A double knockout of the *MyoD* and *Myf-5* genes in mice leads to the absence of myoblasts as well as muscle fibers, which suggests that the expression of at least one of them is necessary for myogenic lineage specification (Rudnicki et al, 1993). Targeted inactivation of the myogenin gene in mice results in a very low number of myofibers, which results in perinatal death due to a defect in development of the

diaphragm (Nabeshima et al, 1993). Targeted inactivation of *MRF*-4 in mice causes upregulation of *myogenin* in addition to rib defects with a range of severity depending on the expression of *Myf-5* (Zhang et al, 1995; Patapoutian et al, 1995).

Myocyte Enhancer Factor 2 (MEF2):

MEF2 proteins are nuclear phosphoproteins that were originally identified as myocyte-specific binding factors induced when myoblasts differentiated into myotubes (Gossett et al, 1989). Four genes encode the MEF2 family of transcriptional regulators, each of which gives rise to multiple proteins via alternative mRNA splicing (Pollock and Treisman, 1991; Yu et al, 1992; Leifer et al, 1993; McDermott et al, 1993; Breitbart et al, 1993; Martin et al, 1993). The MEF2 gene products, designated MEF2A, MEF2B, MEF2C, and MEF2D, are expressed in overlapping patterns during embryogenesis and in adult tissue (McDermott et al, 1993). In the mouse embryo, MEF2C protein is the first member of the MEF2 family to appear in the somite at day 9.0 pc and is expressed during differentiation of skeletal and cardiac muscle (McDermott et al, 1993). Other *mef2* genes are expressed after birth ubiquitously, while MEF2C is restricted to skeletal muscle, brain, and spleen (McDermott et al, 1993).

MEF2 proteins belong to the MADS [minichromosome maintenance 1 (MCM1), Agamous, Deficiens and serum response factor (SRF)] -box family of transcriptional regulators. The MADS-box mediates homo- and heterodimer DNA-binding of MEF2 proteins to the consensus nucleotide site, CTA(A/T)$_4$TAG

(Gossett et al, 1989;Pollock and Treisman, 1991). Immediately C-terminal to the MADS-box is a 28 amino acid region referred to as the MEF2 domain. This region is unique to the MEF2 family and has been demonstrated through deletional analysis to be necessary for DNA binding affinity, cofactor interaction and dimerization (Molkentin et al, 1996). MEF2 gene products are highly homologous within the MADS domain and the adjacent MEF2 domain. However, the carboxy terminal domains of the MEF2 proteins are very different and have been shown to be transcriptional activation domains (Molkentin et al, 1996;Yu, 1996).

The promoters and enhancers of many muscle-specific genes contain the consensus binding site for MEF2 proteins, which have the ability to combine with other transcription factors to activate or repress transcription from target genes. MEF2 proteins are cofactors that associate with MRFs through direct physical interaction to synergistically activate muscle gene transcription (Molkentin et al, 1995;Kaushal et al, 1996). The myogenic bHLH factors and MEF2 require interaction between their DNA-binding domains in order to cooperatively activate transcription (Molkentin et al, 1995). However, activation of transcription by these two factors requires only one of the two to be bound to DNA (Molkentin et al, 1995). The conserved “myogenic residues”, Alanine 114 and Threonine 115, in the basic domain of MyoD are required for transcriptional activation signals in cooperation with MEF2 proteins (Black et al, 1998). When the basic domain of MyoD was mutated in this region, muscle differentiation did not occur. The inability of these mutants to activate myogenesis was overcome by substituting

MyoD or MEF2 transcriptional activation domains with VP16 transcriptional activation domains. This finding suggests that bHLH-MEF2 interaction can be uncoupled from transcriptional activation. In addition, it proves that the conserved amino acid residues in the basic region of the MRFs are necessary for transcriptional activation signals in conjunction with MEF2 (Black et al, 1998). Stable transcriptional complexes of MEF2 can occur only with MyoD heterodimers, and not with homodimers (Molkentin et al, 1995). This finding suggests that MRF dimer formation with E-proteins is required for cooperation with MEF2 to synergistically activate muscle gene transcription.

Muscle regulatory bHLH proteins and MEF2 have been shown to amplify and regulate each other's expression. Forced expression of myogenin or MyoD in non-muscle cells leads to induction of MEF-2 activity (Cserjesi and Olson, 1991; Lassar et al, 1991). The myogenin gene promoter contains a MEF2 site and an E-box that is essential for muscle specificity and positive autoregulation of myogenin transcription in skeletal muscle cells (Edmondson et al, 1992; Naidu et al, 1995). The MRF4 promoter also has an E-box and a MEF2 site, but is not capable of autoregulation. However, myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis (Naidu et al, 1995).

Several other regulatory circuits exist to activate and repress MEF2. Calcineurin, a serine/threonine protein phosphatase, regulates skeletal muscle differentiation by activating MEF2 and MyoD transcription factors, which increase myogenin expression and induce terminal differentiation (Friday et al, 2003).

Phosphatidylinositol 3-kinase (PI3-kinase) induces phosphorylation of MEF-2 proteins in order to increase skeletal muscle differentiation (Tamir and Bengal, 2000). The phosphorylation of MEF2A and MEF2C by p38-kinase in a manner similar to, but distinct from, PI3-kinase, also allows these factors to activate transcription (Han et al, 1997;Zhao et al, 1999). Skeletal muscle formation is prevented by chemical inhibitors that block p38 and PI3-kinase activity (Li et al, 2000). MEF2 proteins also control their own activity by recruiting histone acetylases and histone deacetylases (HDACs). Histone acetyl transferases are necessary for transcriptional activation of muscle gene expression (Puri et al, 1997). HDACs repress MEF2-dependent genes through physical interaction with the MADS domain of MEF2 (Sparrow et al, 1999).

The importance of MEF2 protein expression for proper skeletal myogenesis has been demonstrated in a variety of ways. Embryonic lethality due to defective cardiac myogenesis results from deletion of the mouse *mef2c* gene (Lin et al, 1997). Targeted disruption of the *Drosophila melanogaster mef2* gene prevents cardiac, skeletal and smooth muscle differentiation (Bour et al, 1995;Lilly et al, 1995). Differentiation of cultured skeletal muscle cells also is impeded by dominant negative MEF2 mutants (Ornatsky et al, 1997).

Activator-protein 1 (AP-1):

AP-1 is a dimeric transcriptional regulator that was originally identified as a 12-O-tetradecanoyl phorbol-13-acetate (TPA)-inducible compound (Lee et al, 1987a; Angel et al, 1987). Three families of proteins have been identified that

participate in AP-1 complex formation: Jun (c-Jun, JunB and JunD), Fos [c-Fos, FosB and Fos-related antigens (Fra)-1 and –2] and activating transcription factors (ATF). Cellular-Jun (c-Jun) was the first component of AP-1 to be identified (Struhl, 1987;Angel et al, 1988; Bohmann et al, 1987; Bos et al, 1988). Thereafter, Fos was identified as a partner of Jun and another component of the AP-1 complex. (Rauscher et al, 1988; Sassone-Corsi, 1988).

All components of the AP-1 complex contain an important binding and dimerization motif known as the basic leucine zipper (bZip) in their carboxy-terminal regions (Bos et al, 1989). This motif contains a basic region immediately amino-terminal to a leucine zipper, which is an amphipathic helix formed by five heptad repeats of leucines (Landschulz et al, 1988). The leucine zipper is required for dimerization, while the basic region offers a contact point for DNA. Dimerization is required for AP-1 DNA binding and also increases nuclear translocation of the transcriptional regulator (Halazonetis et al, 1988; Smeal et al, 1989; Chida et al, 1999). Domain-swapping experiments wherein the bzip region of Fos was replaced with that of the yeast transcriptional activator, GCN4, determined that non-leucine residues within the zipper motif determine the specificity of the leucine zipper pairs (Sellers and Struhl, 1989). Members of the Jun family of proteins can form homodimers, although heterodimer formation with the Fos family is more stable and has an increased affinity for the AP-1 DNA binding site (Allegretto et al, 1990; Halazonetis et al, 1988; Nakabeppu et al, 1988; Smeal et al, 1989;Ransone et al, 1990). However, the Fos family of proteins is able to only form stable heterodimers with the Jun proteins. Jun also

is capable of binding with other transcriptional regulators such as members of the ATF/CREB family (Chatton et al, 1993; Hai and Curran et al, 1991). The ability of Jun to dimerize with diverse partners may allow for modulation of target gene specificity. In addition to preference for a dimerization partner, AP-1 also displays specificity for consensus AP-1 DNA binding sites depending on dimer composition. Jun homodimers and Jun-Fos heterodimers prefer the consenseus nucleotide sequence TGACTCA, which is known as the TPA-response element (TRE) (Angel et al, 1987; Lee et al, 1987a). The cyclic AMP response element (CRE) with the consensus nucleotide sequence TGACGTCA is preferred by Jun-ATF heterodimers (Lee et al, 1987b; Montminy et al, 1987; Jiang et al, 1998).

The amino-terminal portion of the Jun proteins contains the main transcriptional activation domain (Angel et al, 1989). Although c-Fos and Fos B have potent transactivation domains, Fra-1 and Fra-2 do not (Suzuki et al, 1991;Wisdom and Verma, 1993). The TAD in the components of AP-1 has been shown to be responsible not only for transcriptional activation and stimulation of DNA synthesis, but also the transformation property of this complex (Morgan et al, 1993).

Gene targeting experiments of the different components of AP-1 support the idea that each component has a different function in vivo. *c-Jun* null mutations are embryonic lethal due to hepatic failure showing that *JunB* and *JunD* are unable to compensate for the loss of expression (Hilberg et al, 1993; Johnson et al, 1993). Mutant *JunB* mice are embryonic lethal due to defective placentation (Schorpp-Kistner et al, 1999). *JunD* null mice display defects in

male reproduction, hormone regulation and spermatogenesis (Thepot et al, 2000). *c-Fo*s mutant mice are viable, but exhibit severe osteoporosis as well as defective hematopoeisis (Johnson et al, 1992; Wang et al, 1992). Targeted inactivation of *FosB* leads to mice that are viable, but have a defect in nurturing their young (Brown et al, 1996). Fra-1 mutant mice died mid-gestation due to a defect in placental vascularization (Schreiber et al, 2000). A knockout mouse of *fra-2* has not been created.

Although the physiological role for AP-1 during skeletal muscle development is not completely understood, many studies have shown AP-1 to be a negative regulator of myogenesis. c-Fos inhibits the formation of skeletal muscle by repressing both the expression and activity of MyoD (Lassar et al, 1989). Myogenic repression by c-Jun is mediated through direct association of the HLH domain in myogenin or MyoD and the c-Jun leucine zipper region (Bengal et al, 1992;Li et al, 1992). c-Fos and c-Jun also have been shown to inhibit MyoD expression and consequently myogenesis by binding directly to a CRE-like element in the MyoD promoter (Pedraza-Alva et al, 1994).

III. Growth factor regulation of skeletal myogenesis:

Insulin-like growth factor (IGF):

The IGF system, which is critical for growth and development, is composed of IGF-I, IGF-II, the IGF receptors, and the IGF binding proteins (IGFBPs) (Le Roith et al, 2001). Transgenic mice overexpressing a chimeric IGF-I gene exhibit an overall increase in growth (Mathews et al, 1988).

Overexpression of IGF-I in vitro increases muscle size by inducing many muscle-specific genes in vitro, including the myogenic regulatory factors (Florini et al, 1990). Induction of differentiation of skeletal muscle by IGF-I is mediated by an increase in myogenin expression (Florini et al, 1986). The necessity of the IGF axis in vivo has been demonstrated by *IGF-I* null and *IGF-I receptor (IGF-IR)* null mice. Three groups simultaneously reported that *IGF-I* null mice are viable, but exhibit a severe decrease in muscle mass (Baker et al, 1993;Liu et al, 1993;Powell-Braxton et al, 1993). Loss of *IGF-IR* is lethal due to respiratory failure resulting from improperly formed skeletal muscles (Liu et al, 1993). IGF-II also plays an important role in myogenesis by acting through an autocrine loop during development (Florini et al, 1991). IGF-I and IGF-II both have proliferative effects on myoblasts. However, IGF-I has stronger mitogenic activity, which ultimately delays myofiber formation (Ewton et al, 1994). IGF-II overexpression results in more extensive differentiation than IGF-I (Ewton et al, 1987).

In cultured myoblasts, the IGFs have pleiotropic effects because they are capable of inducing proliferation and subsequent differentiation, both of which are mediated through the IGF-1R (Ewton et al, 1987; Quinn et al, 1994). The IGF-1R signals primarily through the mitogen activated protein kinase (MAPK) and phosphitidylinositol-3 kinase (PI3-kinase) cascades (Coolican et al, 1997). The MAPK cascade is thought to be responsible for the mitogenic effects because addition of a chemical inhibitor that interferes with MAPKK activity is inhibitory to proliferation of myoblasts and results in prolific differentiation (Coolican et al, 1997). The regulation of the retinoblastoma (Rb) protein phosphorylation is also

partially responsible for the proliferative effects of the IGFs and suppression of myogenin expression (Rosenthal and Cheng, 1995).

Myogenic differentiation is dependent upon downregulation of MAPK activity (Coolican et al, 1997). Time course studies investigating the effects of IGF-I on myogenesis show a transient decrease in the expression of the MRFs and an increase in the expression of cell cycle machinery, which is followed quickly by an increase in MRF and contractile protein expression (Engert, 1996). Inhibition of the PI3K pathway with chemical inhibitors such as wortmannin or LY294002 effectively blocked differentiation and decreased myogenin expression (Coolican et al, 1997; Kaliman et al, 1996). Further work demonstrated that overexpression of a constitutively active form of PI3K enhances myogenic differentiation and muscle specific protein expression in avian myoblasts (Jiang et al, 1998b).

Fibroblast growth factor (FGF):

Fibroblast growth factors are a family of at least twenty mammalian proteins that have control over cellular proliferation, differentiation and survival (McWhirter et al, 1997;Hoshikawa et al, 1998;Miyake, 1998). All members of the family are related by a highly homologous core sequence of approximately 120 amino acids (Zhu et al, 1991). The FGFs bind to heparin sulfate proteoglycans on the outside of the cell, which present the growth factors to one of a family of four extracellular surface FGF receptors (FGFR) (Faham et al, 1996). Binding of

the ligands to their cognate receptors initiates activation of intracellular signaling cascades by the membrane-bound GTP-ase, Ras (Gilman, 1987).

In myogenic stable cell lines, some members of the FGF family are inhibitory to skeletal muscle development because terminal differentiation cannot occur in the presence of these mitogens. The effects that these growth factors have on myogenesis apparently occur in two ways. First, several studies have shown a mitogenic effect for FGF-1, -2, and -6 on myoblasts (Lathrop et al, 1985;Hannon et al, 1996;Pizette et al, 1996). This is partially due to the upregulation of cyclinD1, which is promotional to cell cycle progression (Rao et al, 1995).

However, other studies have shown that the inhibition to myogenesis caused by basic FGF (FGF-2) is independent of its mitogenic effects (Spizz et al, 1986;Clegg et al, 1987;Seed and Hauschka, 1988). The downregulation of IGF-II expression is thought to contribute to the overall inhibition of muscle formation by FGF-2 (Rosenthal et al, 1991). Treatment of myogenic cell cultures with exogenous FGF-2 has also been shown to downregulate the expression of markers of muscle differentiation and the master regulatory gene MyoD (Vaidya et al, 1989). Another study concluded that FGF-2 prevents transcriptional regulation of myogenin and the acetylcholine receptor, which is a necessary component for muscular contraction (Brunetti and Goldfine, 1990). FGF-2 is thought to exert its effects not only by suppressing the expression of muscle regulatory factors, but also by preventing their activity. The inhibition of myogenin is thought to be due to phosphorylation of a conserved residue in its

basic domain, which results in the inability of the transcription factor to bind DNA (Li et al, 1992). However, this is not the case for all of the myogenic regulatory factors, as MRF4 isolated from FGF-2 treated myoblasts was not phosphorylated on the same conserved threonine residue (Hardy et al, 1993).

Another mechanism through which FGF-2 may inhibit myogenic differentiation is through upregulation of the inhibitor of differentiation (Id) proteins (Chen et al, 1997). Id proteins are helix-loop-helix proteins that bind to E-proteins and act as dominant negative factors to prevent DNA binding and subsequent transcriptional regulation (Norton et al, 1998). The Id proteins indirectly inhibit binding of MRFs to E-boxes and prevent transcriptional activation of muscle specific genes (Benezra et al, 1990).

Transforming growth factor beta (TGFβ):

The TGFβ superfamily is made up of a diverse group of over thirty polypeptides that includes TGFβs, bone morphogenetic proteins (BMPs), and activins. Members of this family are important regulators of cell proliferation and differentiation, embryonic patterning and organ formation, and tumor suppression (Wang et al, 1988). For instance, TGFβ may cause growth arrest or proliferation in the same cell depending on the presence or absence of oncogenic Ras activity in the cell (Oft et al, 1996;Ashcroft et al, 1999). Much information recently has been uncovered regarding downstream signaling pathways activated by TGFβ.

In spite of the diversity of its actions, a seemingly simple model of TGFβ signal transduction has recently emerged. The ligand molecule signals through a

heteromeric serine/threonine kinase receptor complex composed of two receptor types. Receptor activation occurs by growth factor binding to a specific constitutively active type II receptor, which subsequently phosphorylates and activates the GS domain of the inactive type I receptor (Souchelnytskyi, 1996). There are currently five type II and eight type I receptors, although different members of the TGFβ family seem to routinely signal through certain combinations of these receptors (Attisano and Wrana, 2002). While receptor specificity is responsible for some of the diversity of action observed with these ligands, much is accomplished through divergent signaling pathways within the cell. Downstream of the receptor complex, at least two distinct intracellular pathways may mediate inductive signals from the cell membrane to the nucleus (Attisano and Wrana, 2002).

The first of these two pathways involves a family of transcriptional activators known as the Smads. Eight Smads have been discovered in vertebrates, which are categorized as receptor-regulated Smads (R-Smads), common-mediator Smads (co-Smads), and inhibitory Smads (I-Smads) (Attisano and Wrana, 2002). Upon receptor activation, an R-Smad is recruited to the complex with the assistance of the Smad anchor for receptor activation (SARA) (Tsukazaki et al, 1998). Smads 2 and 3 act downstream of TGFβ and activin, while Smads 1and 5 relay the BMP 2 and 4 responses (Macias-Silva et al, 1996; Kretzschmar et al, 1997). The type I receptor phosphorylates the R-Smad, which is then released by SARA (Tsukazaki et al, 1998). En route to the nucleus, the R-Smad associates with Smad 4, a Co-Smad that is necessary for assembly of

the transcriptional activation complex (Shi et al, 1997). When the R-Smad/Co-Smad transcriptional complex enters the nucleus, it binds to the target gene's promoter sequence and activates transcription at the Smad binding element (SBE) (Attisano and Wrana, 2002). Smads also are capable of complexing with other transcription factors, co-activators, and co-repressors in the nucleus to induce transcription from a target gene. Although Smads are capable of activating transcription on their own, the activation is very weak due to low affinity DNA binding (Shi et al, 1998). Also, such a simple model of signaling requires cross talk with other signaling pathways in order to elicit the complex effects seen with TGFβ family members. For instance, the Smad 3/4 complex can interact with the transcription factor, activator protein-1 (AP-1) to synergistically strengthen transcriptional activation (Liberati et al, 1999). CBP/p300 also complexes with Smads to induce transcription of target genes (Janknecht et al, 1998; Pouponnot, 1998). Conversely, Smads 6 and 7 have been identified as inhibitors of Smad transcriptional activation by competing for binding to the type I receptor (Imamura et al, 1997; Hayashi et al, 1997). R-Smads are reputed to be necessary components of TGFβ signaling in many different cell types, as shown by knockout studies in mice (Weinstein et al, 1998; Yang et al, 1999; Galvin et al, 2000). For example, mutations and deletions in Smad 4 are often associated with tumorigenesis, suggesting activity as a tumor suppressor (Hahn et al, 1996).

Some members of the TGFβ superfamily are capable of activating mitogen activated protein kinase (MAPK) pathways (Axemann et al, 1998). The activation of MAPK may involve TGFβ-activated kinase 1 (TAK1) (Yamaguchi et al, 1995;

Shibuya et al, 1998). While the exact details of the direct activation of TAK1 by TGFβ family members remains largely unknown, TAK1 binding protein 1 is suspected to play a role in the activation of this kinase pathway member (Shibuya et al, 1996). Downstream of TAK1, a rapid and transient upregulation of the MAP kinase (MAPK) family members, p38 and c-jun-N-terminal kinase (JNK), upon TGFβ binding to its cognate receptor is well established (Hannigan et al, 1998;Yamaguchi et al, 1998;Engel et al, 1999). The JNK pathway phosphorylates and thereby activates the AP-1 component c-jun, while the phosphorylation target of the p38 kinase pathway is ATF-2. Both of these factors are capable of cooperating with Smad transcriptional complexes to increase transcriptional activation of target genes.

TGFβ is a potent inhibitor of myogenesis and several theories exist to explain this phenomenon (Florini et al, 1986; Olson et al, 1986; Massague et al, 1986). TGFβ-treated fibroblasts that constitutively express myogenin do not exhibit upregulation of the dominant inhibitory protein, Id, which suggests that TGFβ and FGF utilize different mechanisms in order to inhibit fiber formation (Brennan et al, 1991b). The same study concluded that treatment of these cells with TGFβ inhibited myogenin transcriptional activity, while myogenin DNA binding activity was unaffected. Similarly, the protein expression and function of MyoD also is inhibited by TGFβ (Vaidya et al, 1989). Because TGFβ does not affect the binding activity of the MRFs, it is possible that the inhibitory effects of this growth factor are partially exerted through repression of cofactor interaction. Inhibition of myogenesis by TGFβ has been reported to be dependent upon cell

density and translocation of MEF2 to the cytoplasm, which would effectively prevent interaction of this cofactor with MRF proteins (De Angelis et al, 1998). Nuclear overexpression of MEF2 reinstates the differentiation program in these cells. Smad 3 associates with MyoD and interferes with heterodimerization of MyoD to an E-protein. This, in turn, prevents binding of the MRF to an E-box and initiation of transcription (Liu et al, 2001).

Myostatin (GDF-8) is a member of the TGFβ family that also negatively regulates skeletal muscle mass (McPherron et al, 1997a). This is evidenced by the myostatin knockout mouse, which has a large increase in skeletal muscle mass due to both hypertrophy and hyperplasia (McPherron et al, 1997a). In cattle, mutations in the myostatin gene are responsible for the "double-muscling" effects seen in breeds such as the Belgian Blue and Piedmontese (Kambadur et al, 1997, McPherron et al, 1997b; Grobet et al, 1997). Recent evidence suggests that the expression of MyoD is increased in the fetuses of cattle that are phenotypically double-muscled, which may be due to the absence of biologically active myostatin in these animals (Oldham et al, 2001).

Myostatin signals through the Activin type II receptor resulting in the downstream activation of Smad 3 (Lee and McPherron, 2001;Langley et al, 2002). The use of recombinant myostatin in cultured myoblasts resulted in cell cycle arrest and inhibition to proliferation (Thomas et al, 2000). Another study found that myostatin not only inhibited cellular proliferation, but also prevented DNA and protein synthesis in myogenic cells (Taylor et al, 2001). Experiments in which myostatin was transiently transfected into mouse myoblasts revealed

that cell death associated with differentiation was reduced, which most likely involves upregulation of $p21^{cip1}$, a cyclin-dependent kinase inhibitor (Rios et al, 2001). Muscle cells stably expressing myostatin also exhibit a dramatic decrease in myogenic differentiation, resulting from a reduced level of MyoD and myogenin and decreased activity of muscle creatine kinase (Rios et al, 2002;Langley et al, 2002). Therefore, negative regulation of skeletal muscle formation by myostatin occurs not only through inhibition of proliferation, but also through blocking terminal differentiation.

IV. Control of skeletal myogenesis by signaling cascades

Ras:

Several growth factors are known to activate the small, membrane localized GTPase known as Ras, which is involved in growth control (Boguski and McCormick, 1993;Fieg, 1993). The three genes that encode the *ras* family of proteins are Ha-*ras*, K-*ras*, and N-*ras* (Lowy and Willumsen, 1993). Upon growth factor binding to a receptor tyrosine kinase, autophosphorylation of the receptor occurs, which recruits Grb2-Sos to the Src homology (SH2) domain. Sos, which is now in close proximity to Ras, catalyzes the exhange of a GDP for a GTP (Joneson and Bar-Sagi, 1997). Activated Ras proteins are capable of inducing many downstream signaling pathways that have multiple effects on skeletal myogenesis (Mitin et al, 2001).

Constitutive activation of Ras family members has been etiologically linked to several types of tumors (Webb et al, 1998). In cultured myoblasts, overexpression of oncogenic forms of Ras is both phenotypically transforming and inhibitory to terminal differentiation (Olson et al, 1987;Konieczny et al, 1989). Oncogenic Ras expression increases autocrine growth factor secretion, which results in deregulated cell growth (Weyman and Wolfman, 1997). Another mechanism utilized by oncogenic Ras in order to prevent myogenic formation is through inhibition of the expression of MyoD and myogenin (Lassar et al, 1989;Konieczny et al, 1989). Several studies also indicate that interference with the activity of the MRFs contributes to the block to myogenesis incurred by activated Ras (Kong et al, 1995;Ramocki et al, 1997). However, these factors are still capable of binding DNA and inducing muscle specific gene activation (Kong et al, 1995). This finding suggests that MRF inactivation alone is not likely to be the cause of the block to skeletal myogenesis caused by Ras.

The effects of the membrane-bound Ras proteins are mediated through the activation of intracellular kinase cascades, which send signals to the nucleus through a series of steps involving phosphorylation (Joneson and Bar-Sagi, 1997). Ultimately, these cascades result in the phosphorylation and subsequent activation of transcriptional regulators. Two of the primary cascades are responsible for transmitting signals from Ras are the phoshatidylinositol 3 (PI3)-kinase cascade and the mitogen-activated protein kinase (MAPK) cascade (Hunter, 2000).

PI3-kinase:

The family of PI3-kinase enzymes phosphorylates the inositol head of phosphoinositides (Vanhaesbroeck et al, 2001). The PI3-kinase pathway is directly activated by Ras in a GTP-dependenta manner upon binding of IGF-I to its cognate receptor (Rodriquez-Viciana et al, 1994). Several experiments have suggested that PI3-kinase may be the primary signaling pathway that leads to the terminal differentiation of skeletal muscle (Coolican et al, 1997;Kaliman et al, 1996; Jiang et al, 1998a; Kaliman et al, 1998). A constitutively active form of PI3-kinase serves to greatly increase myofiber formation in primary chick myoblasts (Jiang et al, 1998). PI3-kinase has also been shown to increase the transcriptional activity of MEF2 and the MRF Gal4 fusion proteins (Xu and Wu, 2000). By contrast, inhibition of the PI3-kinase pathway with the chemical inhibitors LY294002 and wortmannin prevents muscle-specific gene transcription as well as terminal differentiation without affecting myoblast proliferation (Kaliman et al, 1996;Jiang et al, 1998). Dominant negative PI3-kinase mutants similarly repress myofiber formation in cultured myoblasts (Jiang et al, 1998b).

Ras-dependent activation of PI3-kinase also results in the activation of Akt, which is a serine threonine kinase (Franke et al, 1995). During muscle cell differentiation, Akt expression is increased, which may function in myocyte survival (Fujio et al, 1999). IGF and PI3-kinase induced muscle formation is inhibited by dominant negative forms of Akt (Jiang et al, 1999). Another finding suggests that Akt directly phosphorylates, and thereby inactivates, Raf which inhibits the mitogenic capacity of this kinase (Zimmerman et al, 1999).

MAPkinase:

Ras is also capable of activating MAPK cascades that result in phosphorylation of one of the members of the MAPK protein family. Although there are more than twenty members of the MAPK family in mammals, the three that are most well understood are c-jun N-terminal kinases (JNKs)/ stess-activated protein kinases (SAPKs), extracellular signal-regulated kinases (ERKs) and p38-kinases (Pearson et al, 2001). Each of these proteins contains a homologous domain with the phosphorylation motif, Serine/Threonine-X (any charged amino acid)-Tyrosine, and is generally the last protein to be phosphorylated in a three-kinase cascade (Pearson et al, 2001).

The p38 MAPK cascade, which is induced by IGF-1, is reputed to play a positive role in skeletal muscle differentiation (Conejo et al, 2002). The upstream activators in this cascade are MAPK kinase 3 (MKK3) and MAPK kinase 6 (MKK6) (Robinson and Cobb, 1997). Induction of MEF2C and MyoD expression by the p38 pathway in cultured skeletal myoblasts has been shown to positively regulate myofiber formation (Zetser et al, 1999;Wu et al, 2000). In vitro overexpression of MKK6, which directly activates p38, increased the transcriptional activity of MyoD during myogenic differentiation in C3H10T1/2 fibroblasts (Zetser et al, 1999). However, in the same study, addition of the chemical inhibitor SB203580, which is specific to the p38 pathway, resulted in differentiation-defective cells and dramatically decreased expression of MEF2 and MyoD. In a similar manner, overexpression of a dominant negative form of an upstream regulator of p38, MKK3, effectively blocks both activation of p38 as

well as myofiber formation (Yeow et al, 2001). Recent evidence suggests that p38 MAPK also acts to inhibit the Raf/MEK/MAPK cascade in order to bring about cell cycle arrest and skeletal myofiber formation (Lee et al, 2002).

While the JNK/SAPKs are activated to some extent by serum and growth factors, the majority of their activation arises from cytokines, hypoxia and various cellular stressors (Derijard et al, 1994). MEKK1 activates MKK4 and MKK7, which are upstream activators of the JNKs in this cascade (Lawler et al, 1998). The end result of JNK signaling is phosphorylation of the AP-1 component, c-Jun (Hibi et al, 1993). Recent studies have shown contrasting effects of the JNK cascade on skeletal myogenesis. Overexpression of Rho, which is a possible upstream activator of JNK results in an increase in the size of muscle fibers in vitro (Takano et al, 1998). In direct contrast to this finding, constitutively active forms of JNK have been shown to cause a decrease in skeletal muscle fiber formation (Gallo et al, 1999).

ERK1/2 is activated downstream of Ras by the Raf family of serine/threonine kinases. Although this family of kinases, which includes A-Raf, B-Raf, and c-Raf-1, is highly homologous, each member of the family is regulated differently (Chong et al, 2003). While Ras is required for activation of all Raf isoforms, B-Raf also requires the GTPase Rap-1 (Vossler et al, 1997). Activated forms of Ras recruit Raf-kinase to the cellular membrane, which causes Raf to undergo a conformational change thereby activating the kinase activity of this protein (Pearson et al, 2001). Raf-kinase is then capable of

inducing the MAP/ERK kinase (MEK) 1/2, which in turn activates ERK1/2 (Crews et al, 1992; Nakielny et al, 1992).

Activation of ERK1/2 proteins has been shown to have contrasting effects on skeletal myogenesis. Some evidence suggests that sustained MAPK activity plays a positive role in skeletal muscle differentiation. Overexpression of a constitutively active form of MEK is capable of restoring differentiation to myogenic cells that were maintained in high serum (Gredinger et al,1998). In addition to this, the authors provide evidence that activated Raf or MEK increases MyoD transcriptional activation. Other studies show that a chemical inhibitor to MEK has no effect on muscle differentiation (Conejo et al, 2001). These reports support the idea that MAPK activation is stimulatory to differentiation.

In contrast to these findings, many other studies contend that MAPK is a negative regulator of skeletal muscle differentiation. MAPK activity has been shown to inhibit myofiber formation, but is a requirement for proliferative events (Coolican et al, 1997). Induction of c-Raf-1 in rat myoblasts stably expressing an inducible form of c-Raf-1 resulted in a block to differentiation (Samuel et al, 1999). Overexpression of constitutively activated forms of Raf in vitro resulted in an inhibition to both skeletal muscle differentiation and muscle specific gene transcriptional activation, which was possibly due to increased activation of ERK1/2 (Ramocki et al, 1997;Dorman and Johnson, 1999;Winter and Arnold, 2000). Activated MEK1 exhibited repressive effects on both MyoD activity and terminal differentiation (Perry et al, 2001). Conversely, overexpression of MAPK

phosphatase, which was used to block ERK1/2 activation in mouse myoblasts, resulted in a dramatic increase in myofiber formation (Bennett and Tonks, 1997). In addition to the inhibitory effects on differentiation, constitutively active Raf also induces morphological transformation (Dorman and Johnson, 1999). Although the precise mechanism behind the block to differentiation has not been identified, the activation of the transcription factor AP-1 by this kinase appears to be responsible for the cellular changes in phenotype (Dorman and Johnson, 2000).

The MRFs do not appear to be responsible for the block to differentiation of myogenic cells caused by Raf. Primary chick myoblasts overexpressing activated Raf maintain their ability to express MRFs, although the cells are differentiation-defective (Dorman and Johnson, 2000). The sequestration of MEF-2 in the cytoplasm has been implicated in myogenic inhibition of mouse myoblasts overexpressing Raf-kinase (Winter and Arnold, 2000). By contrast, when overexpressed in chick myogenic cells and C3H10T1/2 mouse fibroblasts inhibited by activated Raf-kinase, MEF-2 protein was detected in the nucleus and still could not overcome the inhibitory effects of this kinase on differentiation (Johnson et al, 2002). This finding suggests that MEF-2 sequestration to the cytoplasm is not sufficient to inhibit myogenesis.

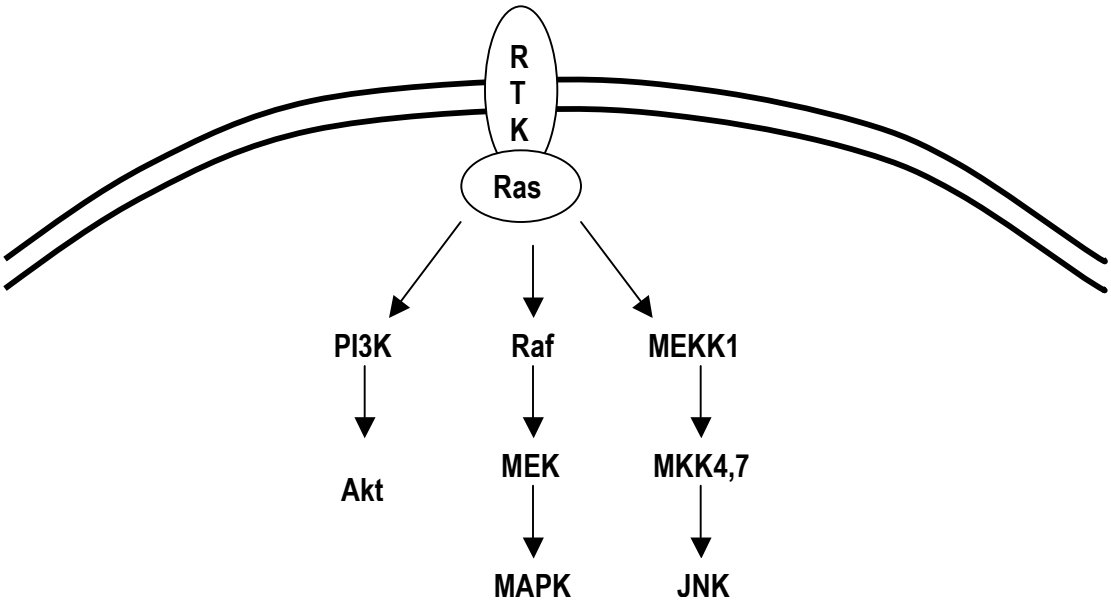

Figure 1.2. MAPK signaling cascades

Central Hypothesis

Initiation of intracellular signaling cascades by growth factor binding precisely controls myogenesis. Raf has been shown to not only inhibit myoblast differentiation by increasing MAP-kinase activity, but also to potently induce myoblast transformation. The ability of these kinases to impede myogenesis is independent from their ability to promote morphological transformation, and remains to be elucidated. We propose that activated Raf-kinase inhibits differentiation by changing the subsets of genes that are normally expressed during skeletal muscle development. The objectives of this work were to identify factors that are secreted by the cell in response to Raf-kinase activation and to identify the downstream targets of the Raf-kinase signaling pathway that contribute to the block to differentiation caused by this kinase.

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Chapter 2

ISOLATION AND CHARACTERIZATION OF CHICKEN TAXREB107, A PUTATIVE DNA BINDING PROTEIN ABUNDANTLY EXPRESSED IN MUSCLE

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I. Abstract

Myogenic regulatory factors (MRFs) are vital transcription factors that act at multiple points during development to establish the skeletal muscle phenotype. This class of muscle-restricted, basic helix-loop-helix (bHLH) proteins acts in concert with additional transcriptional modulators to precisely control muscle gene expression. We have isolated the chicken homologue of Tax responsive element binding protein 107 (TaxREB107). The cDNA is 83% homologous at the amino acid level to human and mouse TaxREB107 and contains a centrally located leucine zipper motif. Northern analysis demonstrated that the gene is expressed in multiple tissues including skeletal muscle. Immunofluorescent staining revealed that the cTaxREB107 protein is located in both the nuclear and cytoplasmic compartments. Distinct localization to the nucleoli supports the evidence that TaxREB107 is a ribosomal protein. Because TaxREB proteins also are implicated in transcriptional regulation, we overexpressed cTaxREB107 in embryonic myoblasts. cTaxREB107 increased troponin I reporter gene activity as well as MRF-directed transcription from a multimerized skeletal muscle E-box reporter gene (4Rtk-luc). However, cotransfection of expression plasmids coding for MyoD and cTaxREB107 did not produce an increase in 4Rtk-luc suggesting that cTaxREB107 enhances myogenic gene transcription through a means independent of a physical association with MyoD. In conclusion, our results define a role for cTaxREB107 during avian myogenesis as a positive modulator of skeletal muscle gene expression.

II. Introduction

The formation of skeletal muscle is controlled, in part, through the coordinated actions of the muscle regulatory factors (MRFs), a class of basic helix-loop-helix transcription factors comprised of *myf-5*, *MyoD*, *myogenin* and *MRF4* (for review see Sabourin and Rudnicki 2000). Gene ablation and *in vitro* mis-expression studies have firmly established the importance of this family of muscle-restricted transcription factors in lineage determination, myoblast proliferation and myocyte differentiation (for review see (Arnold and Braun 1996). However, the MRFs are also capable of interactions with other transcriptional regulators to more precisely direct myogenesis. The prototypical example is the MADS transcription factor, myocyte enhancer factor 2C (MEF2C), which specifically interacts with MRF: E-protein heterodimers to enhance muscle gene transcription synergistically (Francisco and Olson 1999; Molkentin et al 1995; Molkentin and Olson 1996). In a similar manner, muscle-LIM protein (MLP) physically associates with MyoD: E47 heterodimers to improve their DNA binding capacity and increase MRF-directed transcriptional activity (Kong et al 1997). Alternatively, several transcription factors negatively regulate MRF action. For example, the basic leucine zipper (bZIP) oncoprotein, c-Jun, forms protein complexes with MyoD that may account for the inhibition of MyoD-directed transcriptional activity (Bengal et al 1992). By contrast, ZEB, a zinc finger protein interferes with myogenesis independent of the formation of higher order protein complexes with the MRFs (Postigo and Dean 1999).

Tax responsive element binding (TaxREB) proteins were originally identified by virtue of their ability to bind to the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) enhancer (Tsujimoto et al 1991). DNA contact by this class of proteins is accomplished through a centrally located bZIP motif. To date, the TaxREB family includes TaxREB107, TaxREB67/CREB-2/ATF4, TaxREB302, TREB5/XBP-1, TREB7 and TREB36/ATF-1 (Masaki et al 2000; Morita et al 1993; Nyunoya et al 1993; Okuyama et al 1996; Tsujimoto et al 1991; Yoshimura et al 1990). Each of these proteins is thought to play a critical role in mediating the oncogenic activity of the viral protein Tax, the causative agent of adult T-cell leukemia (Brauweiler et al 1995). Since Tax stimulates transcription of viral and cellular genes through a means independent of direct DNA binding, other proteins are required to interact with the HTLV-I enhancer *cis* element and provide a docking interface for Tax (Brauweiler et al 1995).

In a screen to identify genes differentially expressed in Raf-transformed myoblasts, we isolated a partial cDNA homologous to human TaxREB107. Because TaxREB107 proteins have been implicated in transcriptional regulation, we chose to examine the role of this bZIP protein during avian myogenesis. Here we report that cTaxREB107 is abundantly expressed in muscle and that forced expression of the protein enhances skeletal muscle gene expression.

III. Materials and Methods

cDNA Isolation and Sequencing

A partial cDNA for TaxREB107 (284 bp, nt261-545) was isolated from a cDNA library constructed from RNA purified from day 10 (ED10) chicken embryo myoblasts. Three-prime and 5' rapid amplification of cDNA ends (RACE) was used to generate the full-length cDNA. Briefly, a cDNA library was constructed from poly(A) mRNA isolated from ED10 heart tissue according to the manufacturers recommendations (SMART RACE cDNA Amplification, Clontech, Palo Alto, CA). Oligonucleotide primers for amplification of the 5' terminus of TaxREB107 were the gene specific primer, GSP-1 (5'-ACGCTTGCCTCTGTGACGACCAGT), and the kit supplied SMART oligonucleotide primer (Figure 1). The 3' end of the cDNA was obtained in a similar manner using the gene-specific primer, GSP-2 (5'-CCACGTGCAACCATCATCAAACCGGTT), and the aforementioned kit supplied primer. The amplicons were gel purified and cloned into pBS-KS II (Stratagene, La Jolla, CA). Plasmids containing inserts were identified and sequenced along both strands (Davis Sequencing, Davis, CA). The cDNA nucleotide sequences were assembled with DNAMAN (Lynnon Biosoft) and analyzed by BLAST. The cTaxREB107 cDNA sequence has been submitted to GenBANK (AY032864).

Northern Blot Analysis

Total RNA was extracted from embryonic, neonatal, and adult chicken tissues, as well as cultured chick myocytes, using the single-step method developed by Chomczynski and Sacchi (1987). RNA samples were separated through 1.2% agarose gels containing formaldehyde, transferred to Hybond-N+ nitrocellulose membranes (Amersham, Piscataway, NJ) and UV crosslinked. Twenty-five nanograms of a 284 base pair cDNA fragment corresponding to nucleotides 261-545 of cTaxREB107 were labeled with [α-^{32}P] dCTP by random priming (DECAprime II, Ambion, Austin, TX). Hybridization was performed overnight in Ultrahyb buffer (Ambion, Austin, TX) at 42^{o}C using five nanograms of labeled cTaxREB107 cDNA. Blots were washed twice for five minutes each in 2X SSC/ 0.1 % SDS and twice for twenty minutes each in 0.1X SSC/ 0.1 % SDS. Northern blots were exposed to RX X-ray film (Fuji Medical Systems, Stamford, CT) and developed by autoradiography.

Plasmids, Cell Culture and Transfections

The coding sequence of cTaxREB107 was cloned in frame with a multimerized myc-epitope in the vector, pCS2+MTshuttle, followed by subsequent subcloning into the replication-competent avian retroviral vector, RCAS(BP)A [RCAS(A)] (Dorman and Johnson 1999). In a similar manner, the coding sequence of cTaxREB107 was cloned in frame with the carboxyl terminus of the cDNA encoding yellow fluorescent protein to create pEYFP-cTaxREB107

(Clontech, La Jolla, CA). The cDNA sequence of the final plasmids was verified by automated sequencing (Davis Sequencing, Davis, CA).

Primary myoblasts were isolated from ED10 chick hind limbs, as described previously (Dorman and Johnson 1999). The cells were seeded ($2X10^5$ cells/well) on gelatin-coated, 6-well tissue culture plates in Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 2% chicken serum, 1% penicillin/streptomycin and 10 μg/ml gentamicin (Gibco BRL, Grand Island, NY). Cells were transfected one day after isolation by calcium phosphate-DNA precipitate formation (Kong et al 1995). Cultured myoblasts were transfected with 1 μg of the muscle-specific reporter gene plasmid, troponin I luciferase (TnI-luc), and 0.5 μg pEYFP or pEYFP-cTaxREB107 and 0.5 μg CMV-LacZ. Alternatively, myoblasts were transiently transfected with a multimerized muscle E-box reporter plasmid (4Rtk-luc) and the aforementioned mammalian expression plasmids. Cell lysates were prepared 48 hours post-transfection and luciferase and β-galactosidase activity were measured. Luciferase activity was normalized to β-galactosidase activity and the amount of corrected activity directed by the reporter-only was set to 100%.

C3H10T1/2 fibroblasts were cultured in basal Eagle medium (BME) containing 10% fetal bovine serum and 1% penicillin/streptomycin and 10 -μg/ml gentamicin. Cells ($1X10^5$) were transiently transfected with 1 μg of TnI-Luc, 0.5 μg of pEM or pEM-MyoD, 0.5 μg of pEYFP or pEYFP-cTaxREB107 and 50 ng of pRL-CMV (Promega), as described above. Differentiation was induced by

culturing the cells in low glucose Dulbecco's minimal Eagle medium supplemented with 2% horse serum and 1% penicillin/ streptomycin. Forty-eight hours post-transfection, the cells were harvested and the enzymatic activities of luciferase and renilla were quantified. Luciferase activity was normalized to the amount of renilla enzyme activity, an indicator of transfection efficiency. The amount of corrected luciferase reporter gene activity directed by pEM-MyoD was set to 100%.

Immunocytochemistry

To detect cTaxREB107 protein expression, embryonic chick myoblasts or fibroblasts were seeded in 60-mm gelatin-coated tissue culture plates and transfected with 2 □g of RCAS (A) or RCAS (A)-cTaxREB107 proviral DNA. After 72 hours in culture, the cells were washed with phosphate-buffered saline (PBS) and fixed with 4 % formaldehyde for 10 minutes at room temperature. The cells were permeablized and non-specific antibody binding sites were blocked by incubation in PBS containing 5% fetal bovine serum and 0.1% Triton X100. Cells were incubated for one hour at room temperature in anti-myc (9E10 ascites, Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) diluted 1:3,000 in PBS containing 0.5% serum. After extensive washing with PBS, the cells were incubated for 45 minutes with FITC-conjugated donkey anti-mouse (Vector Labs, Burlingame, CA) diluted 1:150 in PBS plus 0.5% serum. Subsequently, the cells were washed with PBS and counterstained with 1 μg/ml 4,6-diamino-2-phenylindole (DAPI) for the detection of nuclear DNA.

Immunofluorescent detection of cTaxREB107 was accomplished with a Nikon TE-200 microscope equipped with epifluorescence. For the detection of myosin heavy chain (MyHC), C3H10T1/2 fibroblasts expressing MyoD were incubated with MF20 hybridoma supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Ames, IA) as described above. Immunoreactive complexes were detected colorimetrically with a peroxidase conjugated secondary antibody (VectaStain ABC, Vector Labs, Burlingame, CA). Representative images were captured to slide film (Kodak Elite Chrome), digitized and compiled with Adobe Photoshop.

Western Blot Analysis

Myocytes transduced with RCAS(A) or RCAS(A)-cTaxREB107 were washed in PBS and lysed with 25 μl of 4X SDS-PAGE sample buffer. Cellular lysates were sonicated briefly and heated at 95 C for 5 minutes. Ten micrograms of total cellular proteins were separated by electrophoresis through a 12% SDS-polyacrylamide gel and transferred to nitrocellulose. The blot was incubated overnight at 4 C in blocking buffer [5% nonfat dry milk in 10 mM TRIS, pH 8.0, 150 mM NaCl, 0.1% Tween-20 (TBS-T)], followed by a one-hour incubation at room temperature in anti-myc (9E10) diluted 1:10,000 in blocking buffer or anti-MyHC hybridoma supernatant (MF20) diluted 1:4 in blocking buffer. After extensive washing with TBS-T, the blot was incubated for 45 minutes with peroxidase conjugated anti-mouse antibody (Vector Labs, Burlingame, CA) diluted 1:10,000 in blocking buffer. After extensive washing with TBS-T, the immune complexes

were visualized by chemiluminescence and exposure to X-ray film (Amersham, Piscataway, NJ).

IV. Results And Discussion

cTaxREB107 is the avian homologue of human and mouse TaxREB107.

To obtain a full-length cDNA for TaxREB107, 5' and 3' RACE was performed using a combination of gene-specific primers and adapter primers supplied by the manufacturer (Clontech, Palo Alto, CA). The two cDNA fragments representing the five prime and three prime ends were joined at the region of overlap by restriction at the common HindIII site. The full-length cTaxREB107 cDNA was sequenced along both strands and analyzed for the presence of open reading frames (ORF). The complete nucleotide sequence of cTaxREB107, as well as the predicted amino acid sequence of the longest ORF, is shown in Figure 2.1. The avian homologue of TaxREB107 is composed of 994 nucleotides, 897 of which encode the predicted 298 amino acid protein. The coding region is flanked by 25 base pairs of upstream sequence and 72 base pairs of 3' untranslated sequence that includes a polyA tract of 23 nucleotides. Computer analysis of the predicted amino acid sequence reveals the existence of a centrally located leucine zipper motif (Figure 2.1, shaded region). The zipper motif is comprised of 4 leucine residues each separated by 6 amino acids. Comparison of the secondary structures of the avian, human and mouse TaxREB107 proteins demonstrates a strong degree of amino acid conservation (Figure 2.2). The overall degree of protein homology between human, mouse

and chick TaxREB107 is 83%. The largest variation between the three protein sequences is found in the amino terminus with mouse TaxREB107 being the most dissimilar. The unique divergence in the amino terminus suggests that the proteins play distinct functional roles *in vivo*.

cTaxREB107 is abundantly expressed in muscle tissues. Expression of the TaxREB107 gene has been demonstrated in multiple adult mouse tissues and in several neoplastic cell lines (Nacken et al 1995; Ohta et al 1994). To examine the normal tissue distribution of cTaxREB107, total RNA was isolated from ED10 chick tissues and assessed for expression of the putative transcriptional regulator (Figure 2.3A). Northern analysis employing a [α-^{32}P]-labeled cTaxREB107 cDNA demonstrated the presence of a single 1 kb mRNA species in all tissues examined including brain, gizzard, heart, liver, lung and skeletal muscle. While cTaxREB107 appears to be expressed ubiquitously, subtle differences in message levels exist. Ample expression of cTaxREB107 is found in muscle tissues and brain with a reduced level of expression found in liver tissue. Because cTaxREB107 was isolated originally from myogenic cells, we chose to further explore the expression profile of cTaxREB107 in muscle tissues (cardiac, skeletal, smooth). Northern analysis was performed on total RNA isolated from gizzard, heart and skeletal muscle of chick at various ages. Again, copious amounts of cTaxREB107 message were found in the ED10 muscle tissues (Figure 2.3B). The elevated expression was maintained throughout embryonic development and into the early neonatal period. However,

substantial amounts of cTaxREB107 were not detected in the heart, gizzard and skeletal muscles of adult animals (6 weeks). The reduction in cTaxREB107 signal is not a reflection of gel loading differences as probing the blot with ^{32}P-18S revealed comparable amounts of RNA in all lanes. Expression of chick TaxREB107 in proliferating and differentiated primary chick myoblasts also was examined by Northern analysis and determined to be mildly upregulated in rapidly dividing skeletal muscle cells (Figure 2.3C). Collectively, these results suggest that cTaxREB107 is developmentally regulated and may play a critical role during the early growth phases of muscle tissues.

cTaxREB107 is both a nuclear and cytoplasmic protein.

Previous reports suggested that TaxREB107 proteins are putative transcriptional regulators by virtue of their ability to bind a 21 bp *cis* element located in the HTLV-I long terminal repeat (Morita et al 1993). Additionally, the protein was isolated as a ribosome component (Chan and Wool 1996). The ribosomal protein L6 signature motif is centrally located (cTaxREB107 amino acids 192-210) in all known L6 proteins including those from yeast, bacteria, C. elegans and mammals (Chan and Wool 1996; Kenmochi et al 2000). To gain a better understanding of the function of this protein in muscle, we chose to examine the synthesis and localization of cTaxREB107 in embryonic myoblasts. To this end, ED10 myoblasts were transduced with a retrovirus coding for myc-tagged cTaxREB107 and analyzed by Western and immunocytochemistry. As shown in Figure 2.4A, myoblasts transduced with RCAS(A)-cTaxREB107

produce a single protein of approximately 38 kDa. The size of the protein is consistent with the predicted molecular weight (plus epitope tag) and with published reports for human TaxREB107 (Morita et al 1993). Parallel plates of RCAS(A) and RCAS(A)-cTaxREB107 transduced myoblasts and fibroblasts were fixed and processed for immunocytochemistry. Fluorescent detection of myc-immunoreactive proteins revealed that cTaxREB107 proteins localize to both the cytoplasm and nucleus (Figure 2.4B). An intense, discrete fluorescent signal was detected for cTaxREB107 that corresponded with the nucleolus structures within the nucleus (arrows). Ribosomal proteins are transcribed and assembled in the nucleolus structures, thus accounting for the dual compartment immunostaining pattern detected in our experiments. Interestingly, mapping experiments have assigned L6/TaxREB107 to a region in human chromosome 12 that is disrupted in Noonan syndrome (Kenmochi et al 2000). The afflicted individuals often have facial abnormalities, are short in stature and exhibit cardiomyopathies (Noonan 1999). Disruption of ribosomal proteins in *Drosophilae* leads to an abnormal phenotype that includes small size, reduced viability and lower fertility (Lambertsson 1998). This has led to the speculation that disruption of L6/TaxREB107 may account for the aberrant growth and development typified by Noonan syndrome (Lee et al 2000). The rapid development of embryonic skeletal muscle requires an abundance of ribosomes to meet the demands for protein synthesis. Therefore, TaxREB107 proteins may play crucial roles during myogenesis by supporting ribosome biogenesis and protein translation as well as contributing to nuclear regulatory events.

Overexpression of cTaxREB107 increases muscle reporter gene activity.

TaxREB107 proteins have been implicated in the control of gene transcription and their expression is up-regulated in transformed and rapidly dividing cells (Li et al 1999; Xu et al 2000). Because cTaxREB107 expression is modest in myogenic cells *in vitro* and in embryonic skeletal muscle, we hypothesized that the protein may be involved in muscle gene transcription. To further explore this possibility, a mammalian expression plasmid coding for EYFP-cTaxREB107 was cotransfected into ED10 myoblasts with the troponin I luciferase reporter plasmid (TnI-luc). After 48 hours, the cells were lysed and analyzed for luciferase activity. Myocytes transiently transfected with TnI-luc exhibit high amounts of luciferase enzyme activity (Figure 2.5). A significant increase in luciferase activity is found in cells overexpressing cTaxREB107 suggesting the protein is a positive regulator of myogenesis. Because myogenesis is dependent upon MRF function, myoblasts were cotransfected with a multimerized muscle E-box reporter plasmid (4Rtk-luc) and EYFP-cTaxREB107. Under differentiation-permissive conditions, high levels of 4Rtk-luc were evident in EYFP control cells. Similar to the results using a complex muscle-specific reporter, cTaxREB107 significantly enhanced muscle E-box mediated transcription. To further assess the apparent increase in muscle gene expression, lysates of RCAS(A) and RCAS(A)-cTaxREB107 myocytes were analyzed by Western blot using anti-MyHC, anti-desmin and anti-α-actinin (data not shown). Results demonstrate a slight increase in MyHC protein expression with no change in the relative levels of α-actinin or desmin proteins in RCAS-

cTaxREB107 myocytes. These results suggest that cTaxREB107 causes a preferential increase in the expression of subsets of skeletal muscle proteins and does not promote a global increase in skeletal muscle gene expression.

cTaxREB107 does not alter MyoD-directed transcriptional activity.

The approximate 80% increase in 4Rtk-luc activity in the presence of cTaxREB107 suggests that the protein is modulating endogenous MRF function. Recently, it was demonstrated that MyoD physically associates with the viral protein, Tax (Riou et al 2000). Because TaxREB107 also interacts with Tax and this association may be necessary for Tax-mediated transcription (Lenzmeier et al 1999; Morita et al 1993), we reasoned that TaxREB107 may enhance muscle gene expression by a direct association with MyoD. Therefore, C3H10T1/2 fibroblasts were transiently transfected with the multimerized muscle E-box reporter plasmid (4Rtk-luc), pEM-MyoD, pEYFP or pEYFP-cTaxREB107 and pRL-CMV. After 48 hours in differentiation-permissive media, the cells were lysed and analyzed for luciferase and renilla activity. Control cells expressing MyoD drive high levels of reporter gene expression, as expected (Figure 2.6A). However, the level of 4Rtk-luc activity directed by MyoD is not affected by co-expression of cTaxREB107. Immunostaining parallel plates for myosin heavy chain expression were consistent with these results (Figure 2.6B). C3H10T1/2 fibroblasts expressing MyoD contain numerous myofibers when cultured under differentiation permissive conditions. A similar number of MyHC expressing myocytes is found in cells overexpressing MyoD and cTaxREB107. In addition,

the size and gross morphology of the cTaxREB107 myofibers does not differ from control MyoD expressing cells. These results argue that cTaxREB107 increases muscle gene expression in avian myocytes by a mechanism that likely is independent of formation of MyoD: cTaxREB107 complexes. However, these results do not preclude the possibility that TaxREB107 interacts with other MRFs, such as myogenin. Further work is required to resolve the issues of TaxREB107 interactions with nuclear proteins and DNA elements.

Conclusions.

In summary, we have isolated the avian homologue of TaxREB107; a putative DNA binding protein that is ubiquitously expressed in multiple tissues. Our data reveals a developmental pattern of expression in each muscle type examined, and confirms the identification of cTaxREB107 as a ribosomal protein. Although forced expression of cTaxREB107 in primary myoblasts increases muscle gene reporter activity, this phenomenon is most likely independent of cTaxREB107: MyoD complex formation. Taken together, these findings suggest a role for cTaxREB107 in regulation of muscle gene expression during development of skeletal muscle.

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                                                                  M  A  G  E  K  K  A  G  E  K  K  A  G  E  K  K  P  A  Q  K   20
1     acgcggggctttccctccccggaagatggcgggtgagaagaaggcgggtgaaaagaaggcaggcgagaagaagccggcccagaag

                K  V  E  G  K  K  P  A  D  K  K  P  V  E  K  K  A  G  Q  K  K  V  R  E  K  I  K  K  P   49
86    aaggtaggagagaagaaaccagctgacaaaaagcccgttgagaagaaggcaggccagaagaaagtacgtgagaagataaagaagcct

                K  K  Y  H  A  S  R  N  P  V  L  A  R  G  I  G  K  Y  S  R  S  A  M  Y  A  R  K  A  L  Y   79
174   aagaagtaccatgccagccgcaaccctgtcctggcccggggtattgggaaatactcccggtcagccatgtatgccagaaaagcgttgtac

                K  R  K  Y  T  A  P  E  T  K  I  E  K  K  K  K  E  K  P  R  A  T  I  I  K  P  V  G  G  D   109
264   aaacgcaaatacactgctccagaaacaaagatagaaaagaaaaagaaagaaaagccacgtgcaaccatcatcaaaccggttggtggagat

                K  N  G  G  S  R  V  V  K  V  R  K  M  P  R  Y  Y  P  T  E  D  V  P  R  K  L  L  S  H  G  K   140
354   aagaatggaggcagccgtgtggttaaagtccgcaaaatgcctcgctactatccaactgaggatgttcctcgcaagcttttgagtcatggcaaa

                K  P  F  S  Q  H  K  R  R  L  R  A  S  I  T  P  G  T  V  L  I  L  L  T  G  R  H  R  G  K  R   171
447   aaaccattttcccaacacaagaggaggctgcgggcttctattactccaggaactgttctgatcctcctgactggtcgtcacagaggcaagcgt

                V  V  F  L  K  Q  L  G  T  G  L  L  L  V  T  G  P  L  A  V  N  R  V  P  L  R  R  A  H  Q  K  F   203
540   gttgtctttctgaagcagcttggtactggcttgttgcttgttacaggacctctcgctgtcaaccgtgtccctctgcgtagggcccaccagaagttt

                V  I  A  T  S  T  K  V  D  I  S  G  V  K  I  P  K  H  L  T  D  A  Y  F  K  K  K  K  L  R  K  P   235
636   gttattgctacatctaccaaggttgatatctctggagtgaaaattcccaagcatctcactgatgcgtactttaagaagaagaagctgcgtaagccc

                K  H  Q  E  G  E  I  F  D  T  E  K  E  K  Y  E  I  T  E  Q  R  K  T  D  Q  K  A  V  D   264
732   aagcaccaggaaggcgagatctttgacactgaaaaagagaaatatgagataacggagcaacgtaagacagaccagaaggcggtggat

                S  Q  I  L  A  R  I  K  K  V  P  Q  L  R  G  Y  L  R  S  T  F  S  L  S  N  G  V  Y  P  H  K  L   296
819   tcccagatccttgcacgaatcaagaaggtgcctcagctccgtgggtacctgcgctccacattctctctctcaaatggagtctatcctcacaaattg

                V  F  *                                                                                   298
915   gtgttctaagtgttctgaaagcaccacattaaatttgaggggaaaaaaaagccccccccaaaaaaaaaaaaaaaaaaaaaaa
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Figure 2.1. The complete nucleotide and amino acid sequence of cTaxREB107. The cTaxREB107 cDNA was cloned by RACE and sequenced. The numbers on the left and right correspond to the nucleotide and amino acids, respectively. The positions of the primers used for RACE of the 5' and 3' cDNA ends are indicated with arrows. The leucine zipper motif is highlighted. The stop codon is demarcated with an asterisk. The sequence has been deposited with GenBank (accession number AY032864).

Figure 2.2. Multiple amino acid sequence alignment of human, mouse and chick TaxREB107 proteins. The predicted amino acid sequences of human, mouse and chick TaxREB107 proteins were computer aligned (DNAMan). The regions of 100% homology are shaded. Numbers on the right correspond to amino acids. A striking divergence in homology is apparent in the amino terminus of the TaxREB107 proteins.

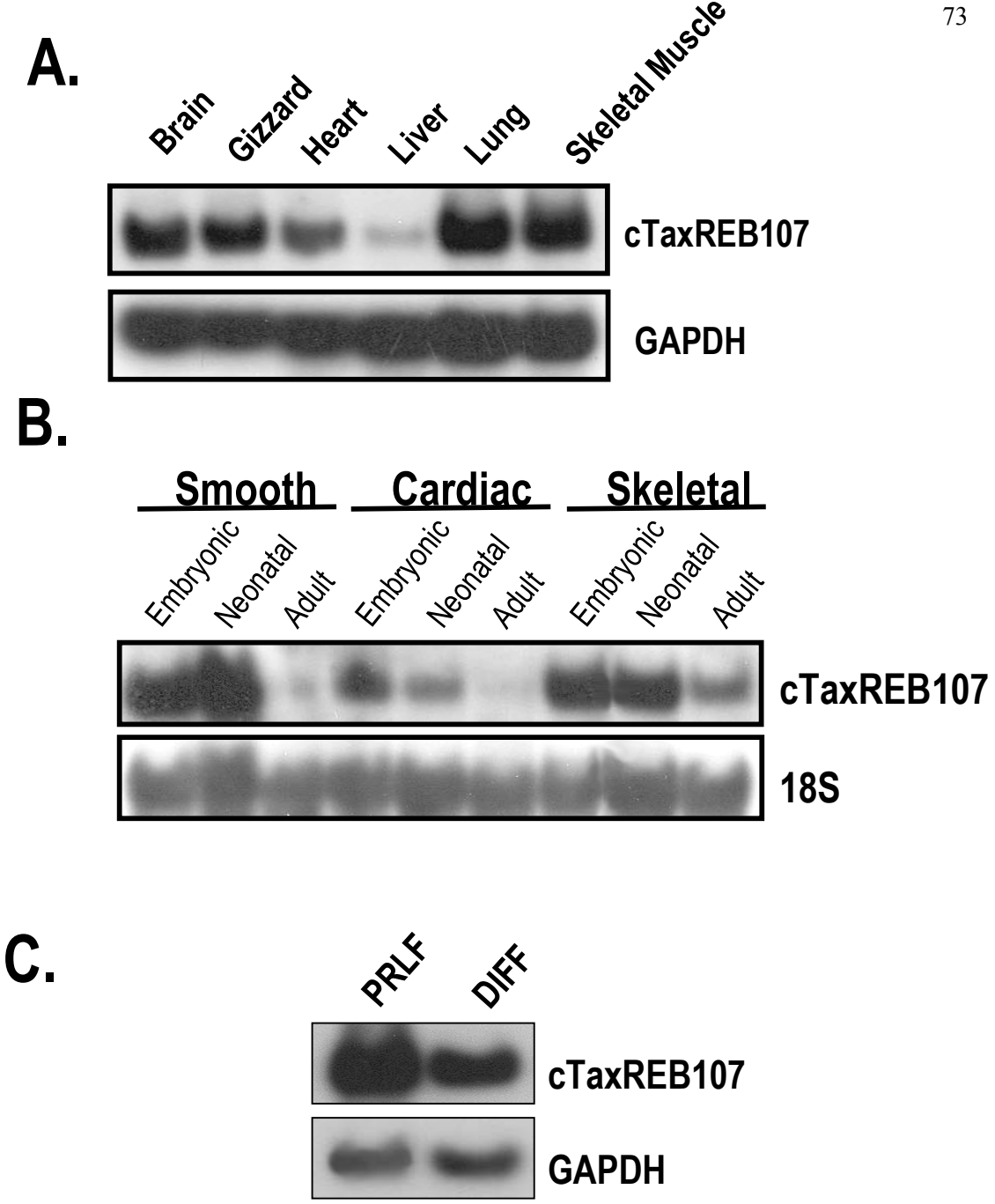

Figure 2.3. Northern blot analysis of cTaxREB107 in various chicken tissues. Total RNA was isolated from the various tissues, as well as from cultured primary chick myoblasts, and hybridized with ^{32}P-cTaxREB107 cDNA probes. Autoradiography demonstrates that cTaxREB107 is expressed in all tissues examined **(A.)**. The expression of cTaxREB107 was examined in muscle tissues isolated from chickens of different ages **(B.)**. Message levels for cTaxREB107 are high in embryonic and neonatal smooth (gizzard), cardiac (heart) and skeletal muscle (hind limb). Low amounts of cTaxREB107 mRNA are found in the adult tissues. Examination of cTaxREB107 expression in both proliferating (PRLF) and differentiated (DIFF) primary chick muscle cells revealed a modest increase in proliferating myoblasts **(C.)**. ^{32}P-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ^{32}P-18S demonstrate equivalent amounts of RNA in all lanes.

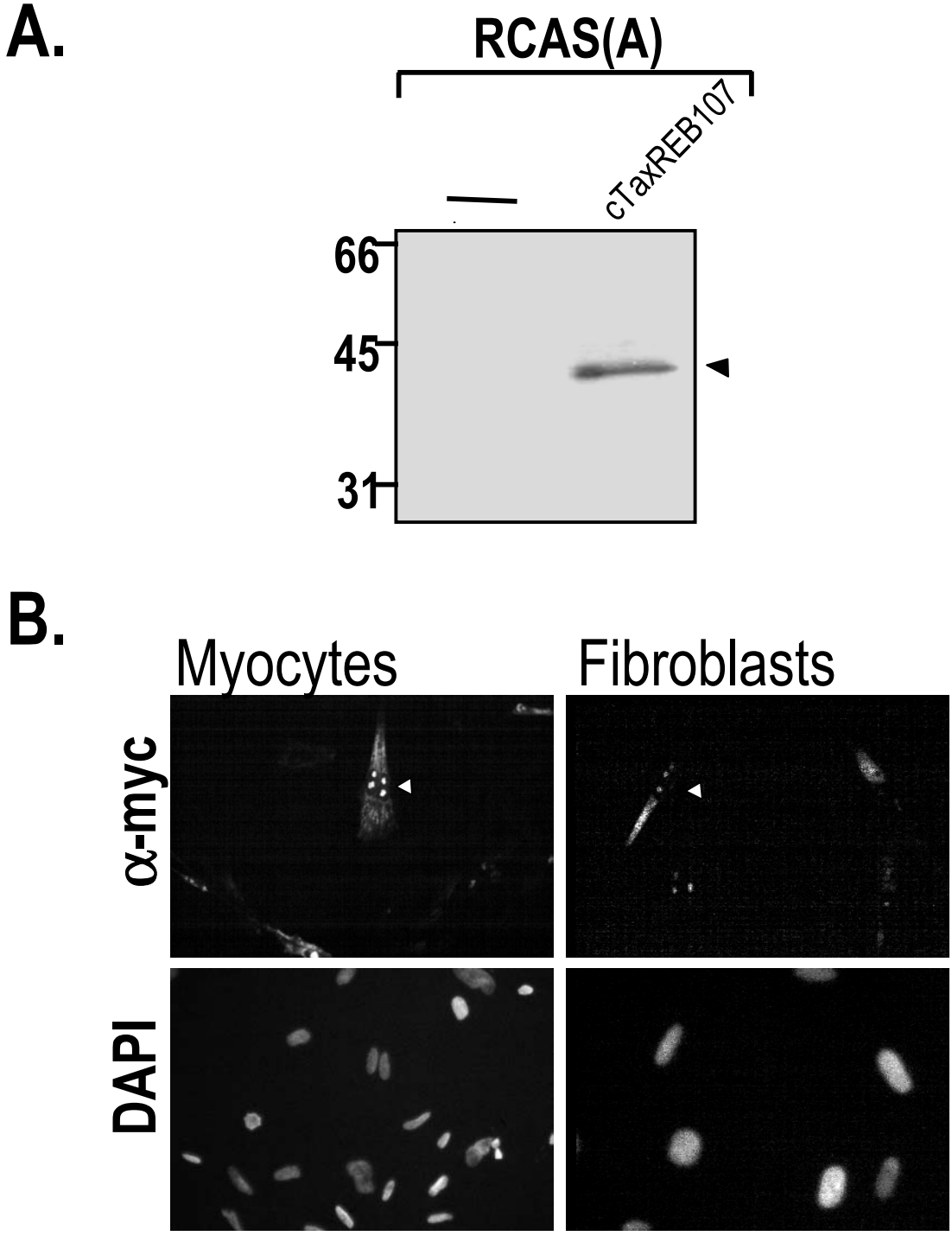

Figure 2.4. cTaxREB107 encodes a protein of 33 kDa that localizes to the nucleus and the cytoplasm. Total protein lysates were prepared from ED10 myoblasts transduced with RCAS(A) and RCAS(A)-cTaxREB107, and equal amounts of proteins were analyzed by Western blot using anti-myc and chemiluminescent detection **(A.)**. RCAS(A)-cTaxREB107 codes for a myc-cTaxREB107 protein of approximately 38 kDa (arrow). ED10 myocytes and fibroblasts were fixed and immunostained with anti-myc and FITC-conjugated secondary antibodies **(B.)**. Immunofluorescent detection demonstrates that cTaxREB107 proteins localize to the nucleolus and cytoplasm (upper panels). Arrows indicate the nucleolus structures. Total nuclei were visualized with 4,6-diamino-2-phenylindole (DAPI) counterstain (lower panels).

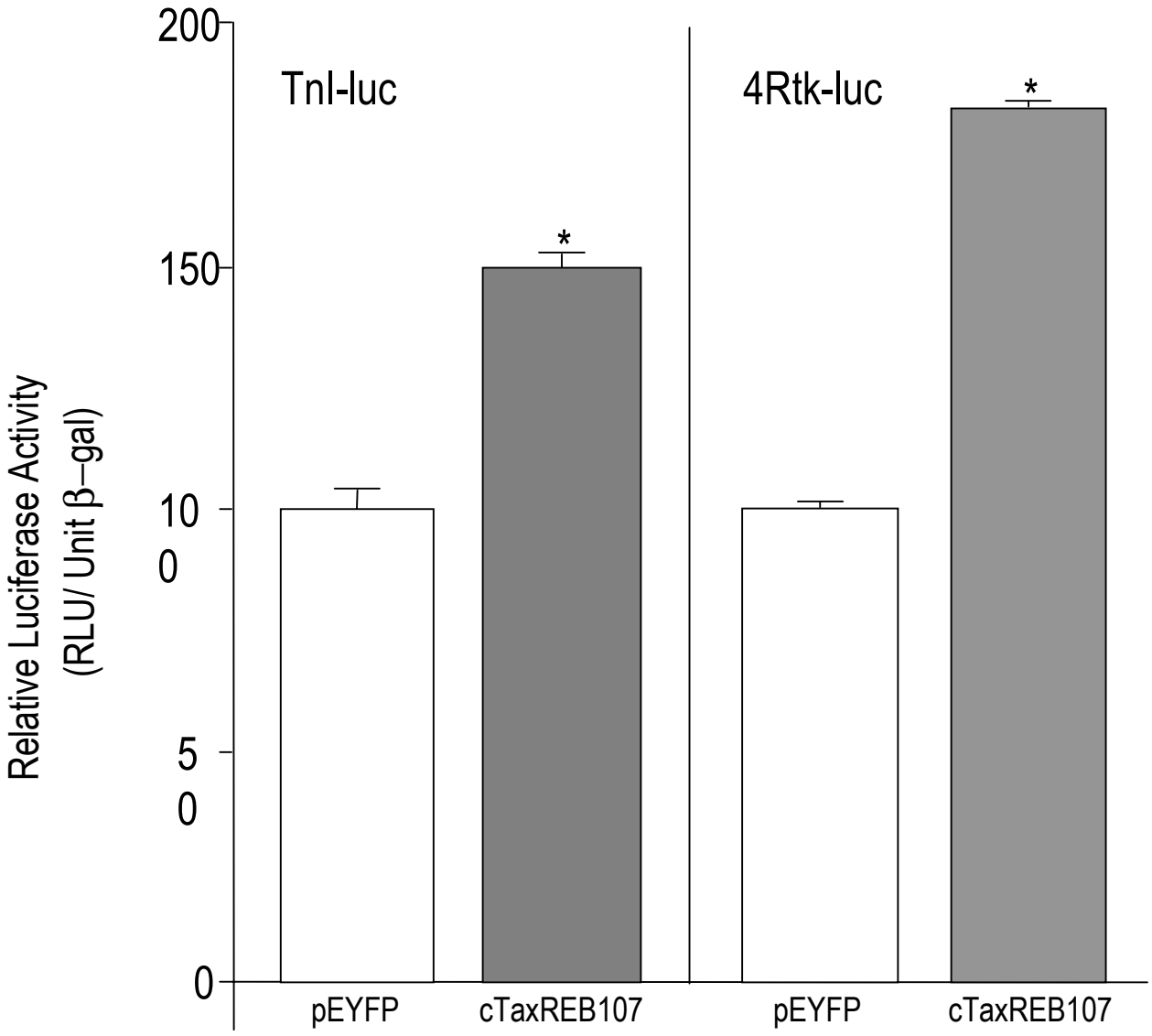

Figure 2.5. cTaxREB107 enhances muscle gene expression. ED10 myoblasts ($2.5X10^5$) were transiently transfected with 2 μg of troponin I-luciferase (TnI-luc) or 4Rtk-luciferase (4Rtk-luc), 0.5 μg of CMV-LacZ and 1 μg of pEYFP or pEYFP-cTaxREB107. After 48 hours in culture, the cells were lysed and luciferase and β-galactosidase activities were measured. The amount of corrected activity directed by pEYFP was set to 100%. Asterisks indicate statistical significance (P<0.05

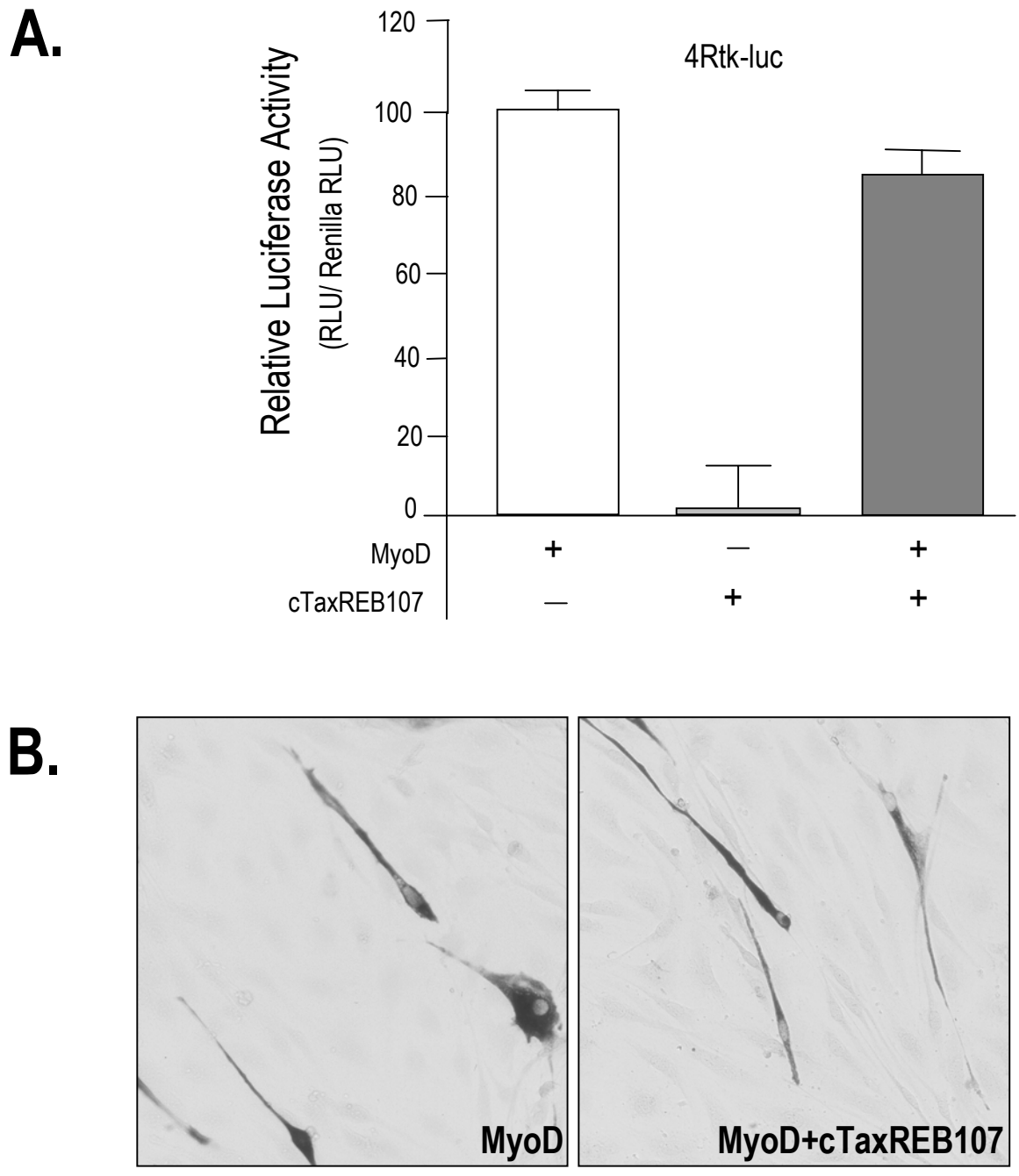

Figure 2.6. cTaxREB107 does not alter MyoD directed transcription or myofiber formation. C3H10T1/2 fibroblasts were transiently transfected with 1 μg 4Rtk-luc, 0.1 μg pRL-CMV and 0.5 μg pEYFP or pEYFP-cTaxREB107 **(A.)**. After 48 hours in culture, the cells were lysed and assayed for renilla and luciferase activity. The amount of corrected luciferase activity directed by MyoD was set to 100%. Immunostaining for MyHC demonstrates equivalent numbers of myosin-containing fibers in MyoD and MyoD plus cTaxREB107 expressing fibroblasts **(B.)**.

Chapter 3

A SHIFT IN AP-1 COMPONENTS CONTRIBUTES TO INHIBITION OF MYOGENESIS THROUGH INTERFERENCE WITH MEF-2 FUNCTION

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I. Abstract

Activated Raf-kinase has a strong negative effect on skeletal muscle fiber formation, although the phenomenon is not well understood. In order to further assess the mechanisms behind the Raf-imposed block to myogenesis, we examined the role of the transcriptional regulation by AP-1 in this event. The precise role of AP-1 in skeletal muscle development is controversial due to the varied effects of the transcription factor. Some studies have found that AP-1 positively regulates myogenesis, while others have reported an inhibitory effect. The differential effects of this transcriptional regulator may be due to the components within the dimer. The main DNA binding components in Raf-induced and TGFβ-treated myoblasts were c-Jun and Fra-2. However, Western blot analysis revealed a dramatic increase in Fra-1, while Fra-2 protein expression was unchanged. Both c-Jun~Fra-1 and c-Jun~Fra-2 inhibited fiber formation and muscle gene transcriptional activation. Interestingly, c-Jun~Fra-1 overexpression appears to inhibit MEF2 function, whereas c-Jun~Fra-2 inhibits the muscle regulatory factors. Our results offer evidence to support the hypothesis that the differential effects of AP-1 are a direct result of subunit composition.

II. Introduction

Skeletal myogenesis is negatively impacted by constitutively active forms of many oncogenes and the growth factors, fibroblast growth factor 2 (FGF2) and transforming growth factor beta (TGFβ). It has been firmly established that overexpression of an activated allele of c-Raf/Raf-1 (Raf) leads to a severe reduction in muscle gene expression and myofiber formation (1-4). In a similar manner, treatment of skeletal myoblasts *in vitro* with TGF-β produces a differentiation defective phenotype that includes loss of mature myocytes and inhibition of muscle-specific gene transcription (5-7). From a molecular standpoint, a contributing factor to repression of myogenesis is disruption of MEF2 function. In both Raf-expressing myoblasts and myoblasts treated with TGFβ, the transcription factor MEF2 is retained in the cytoplasm, which effectively renders the protein inactive (4;8). The myogenic program can be reinstated partially through forced expression of MEF2 in Raf-expressing muscle cells suggesting that additional mechanisms are required for complete loss of muscle formation (9).

TGFβ proteins control cellular decisions mainly through transmission of information via phosphorylation and activation of Smad proteins. The Smad family is a comprised of at least 9 distinct proteins with Smad2, Smad3 and Smad4 present in myogenic cells. In C2C12 skeletal myocytes, TGFβ receptor occupation leads to downstream phosphorylation of Smad3 and subsequent dimerization of Smad3 and Smad4. The Smad complex translocates to the nucleus and interferes with formation of MRF containing heterodimers thus,

preventing muscle gene transcription (10). Overexpression of Smad3 leads to inhibition of morphological and biochemical parameters of myogenesis (10). In addition, forced expression of the inhibitory Smad7 circumvents the detrimental effects of TGFβ treatment in muscle cells demonstrating the importance of this TGFβ specific signaling system (11).

Elevated Raf enzymatic activity leads to the downstream induction of extracellular regulated kinases 1 and 2 (ERK1/2). The transcription factor AP-1 is one of several mediators of ERK1/2 activity and specific components of AP-1 are implicated as regulators of myogenesis. Overexpression of c-Jun or c-Fos leads to suppression of muscle gene expression and myofiber formation (12-14). However, the effects of AP-1 on myogenesis are pleiotrophic and may depend upon the composition of the AP-1 transcription complex. Skeletal myocytes contain both c-Jun:Fra-2 and JunD:Fra-2 complexes, whose appearance is associated with the onset of differentiation (15) JunD is expressed in the myotome coincident with myogenin and JunD containing transcriptional complexes synergize with myogenin to promote muscle formation (13;16). Furthermore, inhibition of c-Jun:Fos in Raf-expressing myoblasts prevents acquisition of the transformed phenotype but does not restore myogenesis suggesting that the Jun dimer partner controls myofiber formation (17).

Due to the striking similarities by which activated Raf and TGFβ inhibit skeletal myogenesis, we examined the interplay between the two signaling systems. Both elevated Raf activity and TGFβ inhibit the entire myogenic program in 23A2 myoblasts including myofiber formation and muscle reporter

gene activation. Gel mobility shift assays indicate that the primary AP-1 DNA binding components found in Raf-expressing and TGFβ treated myoblasts are c-Jun:Fra-2. Interestingly, Western blot demonstrated no significant changes in the levels of Fra-2 protein but a dramatic increase in Fra-1 protein amounts 48 hours after placing the cells in differentiation-conducive conditions. The elevated levels of protein contributed to a shift in AP-1 DNA binding components to include c-Jun:Fra-1. Overexpression of tethered dimers of c-Jun~Fra-1 and c-Jun~Fra-2 in myoblasts resulted in a reduction in muscle reporter gene activity and myofiber formation. Closer examination of the mechanism of inhibition directed by the two AP-1 components indicates that c-Jun~Fra-1 targets MEF2 while c-Jun~Fra-2 impedes MRF function. In summary, the combination of Jun and Fos family proteins dictates the transcriptional response from skeletal muscle genes.

III. Materials And Methods

Plasmids, Cell Culture and Transfections

The AP-1-Luc plasmid, which was used to monitor activator protein-1 transcriptional activity, contains seven copies of the AP-1 enhancer in addition to the firefly luciferase gene (Clontech, Palo Alto, CA). The TnI-Luciferase (TnI-Luc) reporter gene plasmid contains the promoter region from quail fast troponin I (TnI) in addition to the firefly luciferase gene and was used to measure muscle-specific transcriptional activity (18). The 4Rtk-Luciferase (4Rtk-Luc) reporter gene plasmid was used to monitor myogenic regulatory factor (MRF) transcriptional activity and contains a multimerized mouse muscle creatine kinase (MCK)-R E-box in addition to the firefly luciferase gene (19). -228mgn-luc is a reporter construct containing the proximal portion of the chick myogenin promoter, which consists of a MEF-2 site and an E-box (9). -228mEmgn-Luc is a mutated version of the minimal myogenin promoter in which the E-box is inactive. The pRL-tk vector (Promega, Madison, WI) contains the Renilla luciferase gene driven by the thymidine kinase promoter and was used to normalize reporter luciferase activity. The AP-1 tether protein expression plasmids contain the cDNA for JunB, c-Jun, or JunD connected to c-Fos, Fra-1, or Fra-2 by a short linker region (20).

23A2 mouse myoblasts were maintained in Basal Medium Eagle (BME) supplemented with 15% fetal bovine serum (FBS) (Biowhittaker, Walkersville, MD), 1% penicillin/streptomycin and 10 μg/ml gentamicin (Gibco BRL, Grand Island, NY). Differentiation in these cells was induced by replacing the media

with low glucose Dulbecco's Modified Eagle Media (DMEM) supplemented with 2% horse serum, 1% penicillin/streptomycin, and 10μg/ml gentamicin. Ten nanograms per milliliter of transforming growth factor β_1 (TGFβ_1) (R&D Systems, Minneapolis, MN) was added upon induction of differentiation. 23A2Raf[ER]DD2 mouse myoblasts, which stably express an inducible form of the kinase domain of Raf-1, are derived from the 23A2 parental line and were cultured similarly. Constitutive activation of Raf-1 is induced by addition of 2.5 μM 4-hydroxy tamoxifen (4-HT) (Sigma, St. Louis, MO). C3H10T1/2 fibroblasts were maintained in BME supplemented with 10% FBS, 1% penicillin/streptomycin, and 10 μg/ml gentamicin.

All cells were transfected using the calcium phosphate DNA-precipitate method (21). DNA precipitates contained 1 μg of reporter plasmid and 50 ng of pRL-tk. In experiments using the AP-1 tether proteins, 2 μg of each plasmid per precipitate were used in addition to the aforementioned luciferase-reporter plasmids. Following 48 hours in culture, the cells were lysed with luciferase lysis buffer [10% glycerol, 1% Triton-X-100, 25 mM Tris (pH, 7.8), 2 mM dithiothreitol (DTT), and 2mM ethylenediaminetetraacetic acid (EDTA)] and luciferase activities were measured. Reporter activity was normalized to the amount of Renilla luciferase activity present in the samples. The amount of corrected reporter gene activity in the untreated cells was set to one.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear protein extracts were prepared by washing differentiated cultures of 23A2 and 23A2-Raf[ER]DD2 myocytes (grown in the presence or absence of 2.5 μM 4-HT or 10 ng/ml TGFβ_1) in a solution of Tris-buffered saline [10 mM Tris, pH=8.0, and 150 mM NaCl] and 1 mM phenylmethylsulfonyl (PMSF). The cells were scraped into low salt buffer (20mM Tris, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 % TritonX-100, 1mM DTT, 1mM PMSF, and 100 μg/ml Aprotinin) and allowed to swell on ice for 10 minutes. Following centrifugation, nuclei were resuspended in high salt extraction buffer (20 M Tris, 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% TritonX-100, 1 mM DTT, 1 mM PMSF, and 100 μg/ml Aprotinin) and agitated at 4^{o}C for 1 hour. The samples were then centrifuged and the supernatant was removed and quantified (Biorad, Hercules, CA).

One picomole of an AP-1 double-stranded oligonucleotide probe (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with [γ^{32}P]ATP and T4 polynucleotide kinase at 37^{o}C for 1 hour. A spin column containing Sephadex G50 was used to remove free label. Two micrograms of nuclear protein was incubated with 30,000 cpm [γ^{32}P]-AP-1 in addition to 1 μg of a rabbit antibody directed against JunB, c-Jun, JunD, FosB, c-Fos, Fra-1, or Fra-2 (Santa Cruz). Following incubation on ice for 20 minutes, the samples were separated through a 5% non-denaturing polyacrylamide gel in Medium-High Ionic Strength buffer (1 mM Tris, 14% glycine, 1 mM EDTA), dried and exposed to X-ray film (XAR5, Amersham, Piscataway, NJ).

Western Blot Analysis

Differentiated cells were washed in PBS, scraped into 0.5 ml PBS, lysed with Western lysis buffer (20 mM Tris-HCl, pH=7.5, 2 mM EDTA, pH 7.5, 1 mM PMSF, 0.2% Leupeptin, 250 mM Sucrose, 10 mM EGTA), and sonicated. Lysates were assayed for protein concentration (Biorad) and ten micrograms of total cellular lysate in 3X SDS-PAGE buffer (150 mM Tris, 30% glycerol, 3% SDS, 100 mM DTT, 1.5 mg/ml bromphenol blue) was heated at 95^{o}C for 5 minutes and separated through a 12% polyacrylamide gel and transferred to nitrocellulose. The blot was incubated in blocking buffer [5% non-fat dry milk in 10 mM Tris, pH 8.0, 150 mM NaCl, 0.1%Tween-20 (TBS-T)] for 30 minutes at room temperature, followed by incubation for one hour at room temperature in one of the following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) appropriately diluted in blocking solution: JunB (1:500), c-Jun (1:500), JunD (1:200), FosB (1:300), cFos (1:600), Fra-1 (1:300), or Fra-2 (1:300). After extensive washing in TBS-T, the blots were incubated in peroxidase-labeled goat anti-rabbit antibody (Vectastain ABC, Vector Labs, Burlingame, CA) diluted 1:5000 in blocking buffer for 45 minutes at room temperature. Following extensive washing in TBS-T, immune complexes were visualized by chemiluminscence (ECL, Amersham) and exposure to X-ray film.

Immunocytochemistry

Differentiated myocytes were fixed with 4% paraformaldehyde for 15 minutes at room temperature and permeablized for 20 minutes in 0.1% Triton X-

100 in blocking buffer (5% fetal bovine serum in PBS). The cells were incubated in anti-myosin heavy chain (MF-20 hybridoma supernatant, Developmental Studies Hybridoma Bank, University of Iowa, Aimes, IA) diluted 1:4 in blocking buffer followed by extensive washing in PBS. The cells were incubated in 3 μg goat anti-mouse Alexafluor 568/ml blocking buffer (Molecular Probes, Eugene, OR) for 1 hour. Subsequently, the cells were washed with PBS and counterstained with 4,6-diamino-2-phenylindole (DAPI) for the detection of nuclear DNA. Immunofluorescent detection was accomplished with a Nikon TE-200 microscope equipped with epifluorescence.

IV. Results

Activated Raf and TGFβ inhibit 23A2 myogenesis.

Subconfluent 23A2 myoblasts were transiently transfected with TnI-Luc and pRL-tk and cultured in differentiation medium for 48 hours containing 5 ng/ml TGFβ or 1 μM 4HT (23A2Raf). Cell lysates were prepared and luciferase activities measured. Parallel plates were treated analogously and immunostained for myosin heavy chain (MyHC). Induction of Raf/MAPK activity in 23A2Raf myoblasts or treatment of these cells with TGFβ leads to a severe reduction in TnI-Luc activity (Figure 3.1A). The muscle reporter is inhibited to less than 80% of control. In addition to this finding, myoblast fusion is dramatically reduced (Figure 3.1B). In the absence of the growth factor or Raf-kinase induction, 23A2 myoblasts readily fuse into large multinucleated myofibers that initiate transcription from muscle reporter genes. However, myoblasts treated with TGF-β direct a minimal level of TnI-Luc activity and less than 10% of the myoblasts fuse into myosin expressing fibers. These results confirm the strong repressive actions of both TGF-β and the Raf/MAPK signaling axis in 23A2 myoblasts.

Constitutively Active Raf and TGFβ activate AP-1 directed transcriptional activity in 23A2 myoblasts.

Elevated Raf activity is correlated with an increase in AP-1 transcriptional activity in primary chick myoblasts. To determine if TGFβ signaling causes an analogous effect, AP-1 transcriptional activity was measured in 23A2 myoblasts treated with the growth factor. AP-1 luciferase (AP1-Luc) reporter gene and

pRL-tk were transiently transfected into 23A2 and 23A2Raf ER myoblasts. The cells were treated for 48 hours in differentiation medium supplemented with 1 μM 4HT or 5 ng/ml TGFβ. Luciferase activities were measured and corrected for transfection efficiency. As expected, initiation of the Raf/MAPK signaling axis induced a three-fold increase in AP-1 driven transcription (Figure 3.2). These values are similar to those reported previously. Interestingly, treatment of myoblasts with TGFβ elicited a similar response. Levels of AP1-Luc activity were approximately 2.5 fold higher than controls. These results provide additional evidence that TGFβ and activated Raf inhibit myogenesis by comparable mechanisms.

Raf/MAPK activity and TGFβ increase expression of Fra-1.

Activated Raf increases AP-1 directed transcription in skeletal myoblasts (17). TGFβ initiates intracellular signaling through the Smad proteins, which physically associate with AP-1 transcriptional complexes (22). Thus, both negative regulators of muscle formation may serve to impede myogenesis through altered AP-1 function. To this end, we examined the changes in AP-1 protein expression and DNA binding as a function of Raf and TGFβ signaling. In brief, nuclear extracts were prepared from 23A2 myocytes, 23A2RafER myoblasts treated with 1 μM 4HT and 23A2 myoblasts treated with 5 ng/ml of TGF-β for 48 hours in differentiation medium. Two micrograms of total nuclear proteins were analyzed by EMSA using a radiolabeled AP-1 DNA probe and antibodies directed against the specific AP-1 protein components. As shown in Figure 3.3, no detectable DNA binding is apparent for JunB, JunD, FosB, c-Fos

and Fra-1 in the mature myocytes. The primary DNA binding complex appears to be comprised of c-Jun and Fra-2, as described for C2C12 myocytes . Interestingly, upon stimulation of Raf-initiated or TGF-β controlled signaling events, the appearance of an AP-1 DNA binding complex composed of c-Jun and Fra-1 is evident (Figure 3.3 lower panels). The weak DNA binding is supported by a significant increase in the total amounts of nuclear Fra-1 protein. Western blot analysis was performed using antibodies directed against the various Fos and Jun family members and twenty micrograms of nuclear proteins. No significant differences in relative amounts of Fra-2 were found as a result of Raf signaling or TGF-β treatment (Figure 3.4). Induction of Raf phosphorylation events or treatment of the myoblasts with TGF-β resulted in an increase in Fra-1 protein expression by a minimum of ten-fold (Figure 3.4). Interestingly, Western analysis of c-Jun content in control myocytes, Raf-repressive myoblasts and TGF-β treated myoblasts indicated differences in the extent of Jun phosphorylation. 23A2 myocytes and TGF-β treated myocytes contain a single immunoreactive protein band (Figure 3.4, lower panels). By contrast, initiation of Raf kinase results in the appearance of a second c-Jun protein that presumably represents the phosphorylated form. These results provide evidence that inhibition of myogenesis may involve a shift in AP-1 DNA binding components that is reflective of the phosphorylation status of c-Jun.

Inhibition of MyoD-directed myogenesis by different AP-1 complexes.

To clarify the role of c-Jun:Fra-1 and c-Jun:Fra-2 during muscle formation, 23A2 myoblasts were transiently transfected with TnI-Luc or 4Rtk-Luc and

expression plasmids coding for the tethered AP-1 complexes, c-Jun~Fra-1 or c-Jun~Fra-2. Muscle reporter gene activities were measured after 48 hours in differentiation medium and normalized to the amount of pRL-tk activity. Levels of corrected luciferase activity in the control cells were set to 100. The control cells readily activate transcription from both TnI-Luc and 4Rtk-Luc (Figure 3.5). c-Jun~Fra-1 severely reduces the levels of TnI-Luc activity providing support for this AP-1 as a negative regulator of myogenesis. Unexpectedly c-Jun~Fra-2, the primary component in differentiated myocytes, also inhibited transcription from TnI-Luc. Moreover, the chimeric AP-1 protein also suppressed transcription from the minimal E-box promoter indicating that repression is directed at MRFs. The inhibition of myogenesis by the tethered AP-1 proteins extends to the complete differentiation program as both chimeric proteins suppressed myofiber formation by approximately 75% (data not shown). In conclusion, these results document the inability of c-Jun:Fra complexes to synergize with MRFs to promote skeletal myogenesis.

c-Jun~Fra-1 inhibits myogenesis through a mechanism distinct from c-Jun~Fra-2.

To further assess the means by which the two AP-1 complexes disrupt muscle formation, the chimeric proteins were analyzed for their ability to alter transcription from a minimal myogenin reporter (9). The proximal 228 bases of the chick myogenin promoter contain an E-box and a single MEF2 site. Mutation of the E-box leaves a reporter (-228mutEmgn-Luc) that is efficiently activated by MEF2 (9). 23A2 myoblasts were transiently transfected with

–228mgn-Luc or –228mutEmgn-Luc and pRL-tk and allowed to differentiate into myocytes. Cell lysates were analyzed for luciferase activities and muscle reporters were normalized to Renilla. c-Jun~Fra-1 inhibited –228mgn-Luc by approximately 60% (Figure 3.6). –228mutEmgn-Luc, a monitor of MEF2 activity, is inhibited to a similar extent by c-Jun~Fra-1 suggesting that the AP-1 protein impairs MEF2 function. The minimal chick myogenin promoter (-228mgn-Luc) was inhibited in a similar manner by c-Jun~Fra-2. Unexpectedly, myoblasts transfected with c-Jun~Fra-2 retained their ability to initiate transcription from the promoter lacking E-boxes (-228mutEmgn-Luc). This suggests that c-Jun~Fra-2 directly targets the myogenic regulatory factors. In summary, these results extend our observations that AP-1 alters myogenesis in a component-dependent manner.

V. Discussion

Activated Raf-kinase and TGFβ are inhibitory to proper skeletal muscle formation (1;5). Raf-kinase has been shown to increase the transcriptional activation of AP-1 (14;17). Our results indicate that transcriptional activation of AP-1 also is induced when myogenic cells are treated with TGFβ. However, TGFβ previously was thought to primarily signal through Smad transcriptional complexes in order to exert its effects (23). Smad transcription factors are capable of weakly activating transcription alone. However, interaction of the activated Smad transcription factor with AP-1 synergistically enhances transcriptional activation (22; 24). Prevention of MEF-2 localization to the nucleus by both of these pathways has been demonstrated previously (4;8). This suggests that the two pathways are intertwined and may similarly repress skeletal myogenesis.

Protein concentration and activation are both regulators of the transcriptional activity of AP-1 (25). Analysis of protein expression revealed a dramatic increase in the amount of Fra-1 protein present in TGFβ-treated and Raf-overexpressing myoblasts over that of control cells. In addition to this finding, differences in c-Jun phosphorylation in Raf-repressive myoblasts were observed. Previous studies have shown that the stability of c-Jun protein is greatly increased when phosphorylated by c-jun-N-terminal kinase (JNK), which is activated by Raf-kinase (26). Interestingly, the primary DNA binding complex in cells treated with TGFβ as well as those overexpressing Raf was c-Jun:Fra-2. Weak binding of c-Jun:Fra-1 also was observed. Therefore, it is highly plausible

that a shift in the DNA binding components of AP-1 due to the increase in phosphorylation of c-Jun may play a role in the inhibition of myogenesis by Raf-kinase.

AP-1 is generally thought to negatively impact muscle differentiation. However, the cellular effects of this transcriptional regulator are diverse (27). The varied cellular responses to AP-1 most likely depend on the individual components of AP-1, which include Jun and Fos (28-31). For instance, fibroblasts that have been transformed by Ras have increased levels of c-Jun, while levels of JunD are diminished (32). The precise role of AP-1 in skeletal myogenesis is not well defined. In order to further assess the role of specific components of AP-1 in Raf-kinase- or TGFβ-induced myogenic inhibition, we employed the use of various AP-1 components tethered to form a transcriptional complex. Our results show that c-Jun~Fra-1 and c-Jun~Fra-2 tether proteins negatively affect both myofiber formation and muscle specific transcriptional activation. Interestingly, c-Jun~Fra-2 dramatically inhibited transcriptional activity from an E-box reporter, while c-Jun~Fra-1 had no effect on transcriptional activation. This suggests that both AP-1 complexes work in different manners to exert their negative effects on myogenesis. The weak binding of Fra-1 may cause the component to exert a dominant negative effect upon muscle specific transcriptional activity. This could cause interference with the transcriptional activity of MEF2 by forming an inactive transcriptional complex. These results also show that c-Jun~Fra-2 is inhibitory to E-box-directed transcriptional activation. This suggests that the AP-1 complex may be directly interacting with

the myogenic regulatory factors in order to exert its effects on skeletal muscle gene expression.

In view of our data, we propose a model that may explain the role of specific AP-1 components in Raf-mediated myogenic inhibition (Fig. 4.7). Normally, the MRFs combine with MEF2 in order to activate the transcription of the *myogenin* gene (4.7A). The transcriptional activity of c-Jun is increased through Raf-induced phosphorylation of this subunit. TGFβ and Raf-kinase both increase the amount of Fra-1 protein, allowing an increase in the amount AP-1 complexes containing this subunit. c-Jun~Fra-1 complexes inhibit the transcriptional activation of *myogenin* by modulating the activity of MEF2 (4.7B). By contrast, transcriptional complexes containing c-Jun~Fra-2 inhibit the transcription of *myogenin* through interference with the E-boxes, which modulates the ability of MRFs to bind to the *myogenin* promoter (4.7C).

In conclusion, this data supports the hypothesis that specific AP-1 subunits may be responsible for differential activities in the cell. We found that AP-1 is activated similarly in TGFβ-treated and Raf-inhibited myogenic cells. The main binding complexes in these cells are c-Jun~Fra-1 and c-Jun~Fra-2, both of which were shown to negatively regulate skeletal myogenesis. The data presented here is in agreement with other studies that have established AP-1 complexes containing c-Jun as inhibitors of myogenesis (13;14;33). However, c-Jun~Fra-1 was shown to be inhibitory to MEF2 activity, while c-Jun~Fra-2 interfered with MRF activity. Further work will be necessary in order to fully understand the compex nature of the effects of different AP-1 complexes on the differentiation of skeletal muscle.

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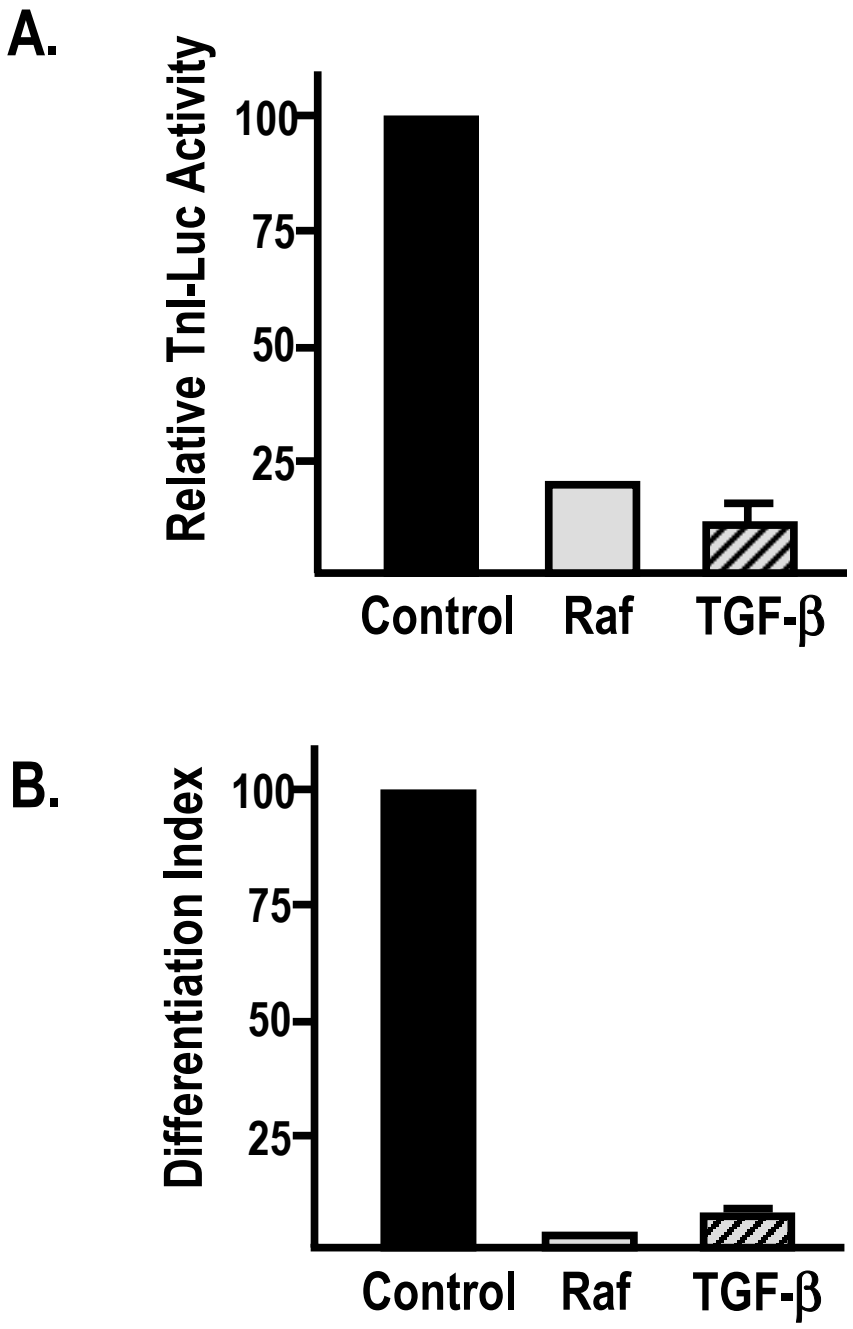

Figure 3.1. Activated Raf-kinase and TGFβ inhibit 23A2 myogenesis. 23A2-Raf[ER]DD2 cells were transiently transfected with 1 μg of TnI-luciferase and 50 ng of pRL-tk and induced to differentiate in the presence of 10 ng/ml TGFβ_1 or 2.5 μM 4-HT **(A)**. After 48 hours in culture, the cells were lysed and assayed for luciferase enzymatic activities. The amount of corrected TnI-luciferase activity in untreated cells was set to 1. The extent of myofiber fusion was assessed in myoblasts that were induced to differentiate in the presence or absence of 10 ng/ml TGFβ_1 or 2.5 μM 4-HT. The cells were fixed with methanol and immunostained for MyHC. Cells were counterstained with DAPI to visualize total nuclei. A differentiation index was determined by calculating the percentage of the total number of cells in multi-nucleate fibers **(B)**.

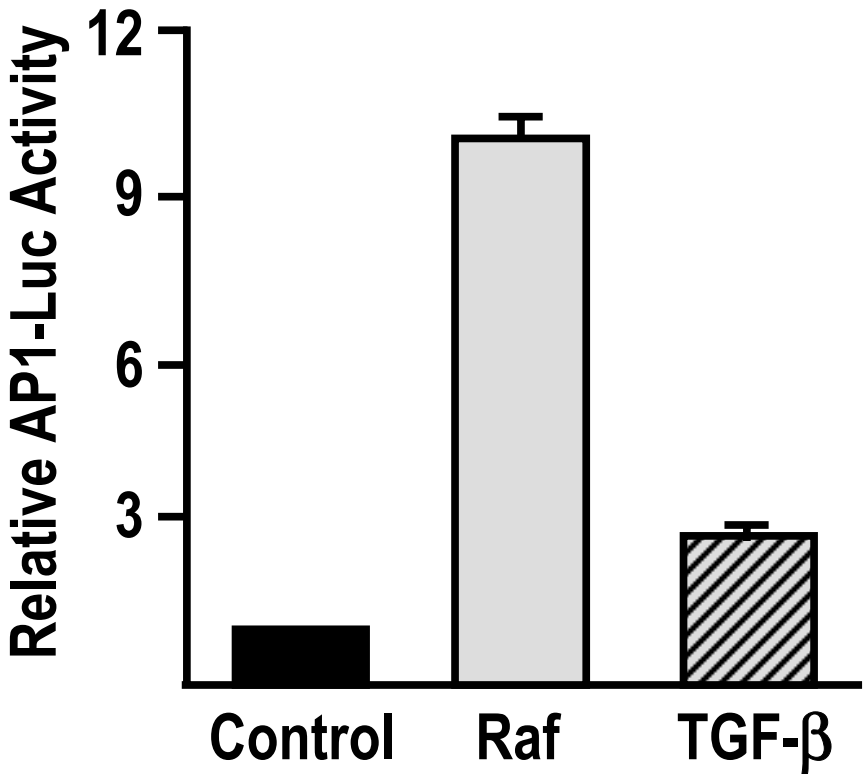

Figure 3.2. AP-1 transcriptional activity in 23A2 myoblasts is increased by TGFβ$_1$ and activated Raf-1. 23A2-Raf[ER]DD2 cells were transiently transfected with 1 μg of AP-1-luciferase and 50 ng of pRL-tk and induced to differentiate in the presence of 10 ng/ml TGFβ$_1$ or 2.5 μM 4-HT. After 48 hours in culture, the cells were lysed and assayed for luciferase enzymatic activities. The amount of corrected AP-1-luciferase activity in untreated cells was set to 1. TGFβ$_1$ and activated Raf-1 both increased AP-1 transcriptional activity.

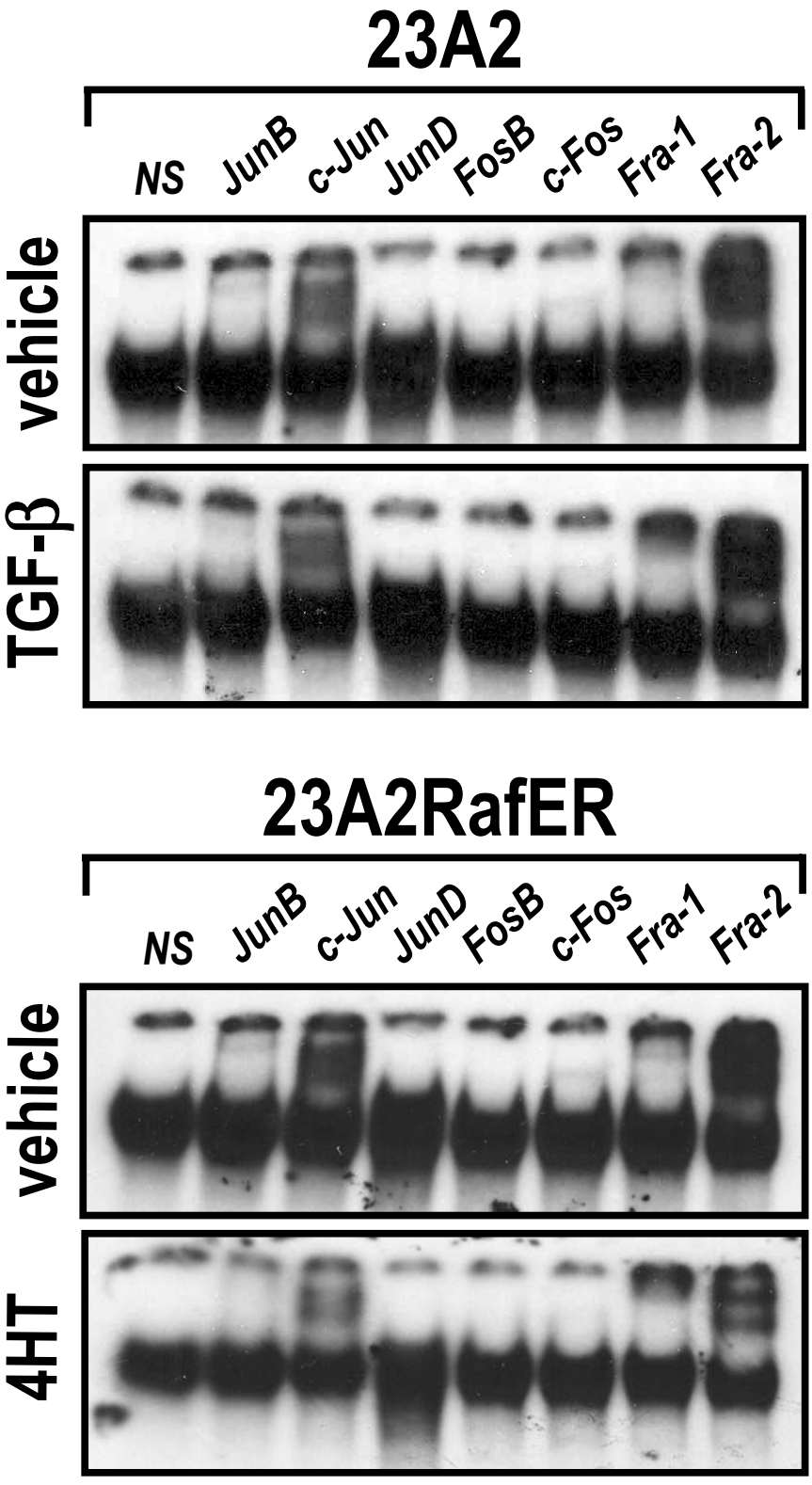

Figure 3.3. Influence of TGFβ_1 and activated Raf-1 on DNA binding activity of AP-1 components in myogenic cells. Two micrograms of nuclear protein were incubated with $\gamma[^{32}P]$-AP-1 probe in the presence or absence of antibodies directed against specific components of AP-1 followed by separation through a non-denaturing gel. Autoradiography demonstrates the presence of c-Jun, Fra-1, and Fra-2 binding complexes.

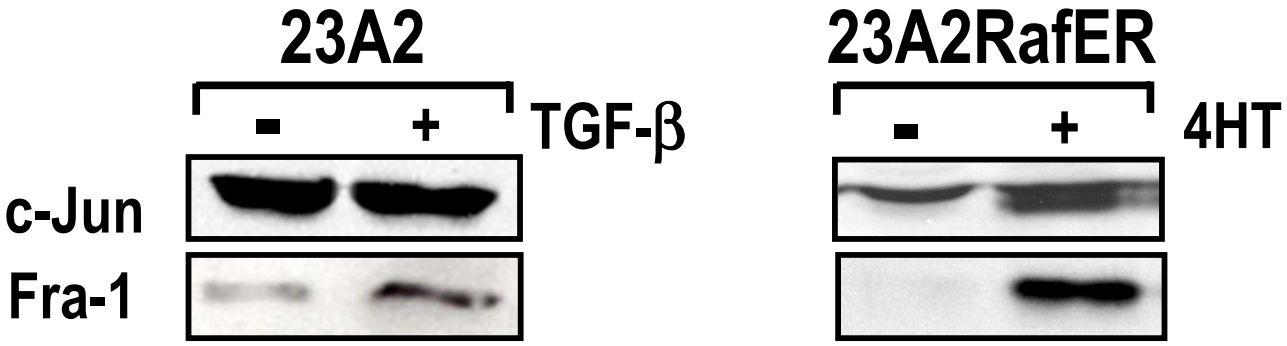

Figure 3.4. Analysis of protein levels of AP-1 components in myogenic cells influenced by TGFβ_1 and activated Raf-1. Ten micrograms of nuclear protein were analysed by Western blot using antibodies directed against components of AP-1. Chemiluminescence reveals an increase in the amount of Fra-1 in cells inhibited by activated Raf-1 as well as in those inhibited by TGFβ_1.

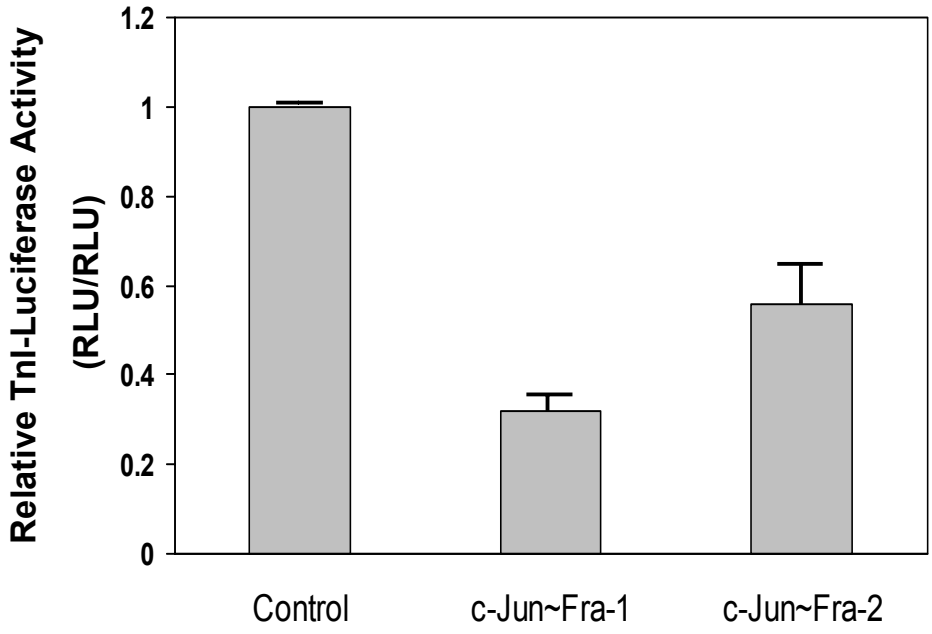

Figure 3.5. cJun~Fra-1 and cJun~Fra2 inhibit myogenic differentiation. 23A2 myoblasts were transiently transfected with 2.5 μg of either cJun~Fra1 or cJun~Fra2 in addition to 1 μg TnI-luciferase or 4Rtk-luciferase and 50 ng pRL-tk, then induced to differentiate upon confluence. Forty-eight hours later, the cells were lysed and assayed for TnI-luciferase or 4Rtk-luciferase and Renilla-luciferase enzymatic activities. Luciferase activity for cells transfected with empty vector was corrected with Renilla activity and set to one.

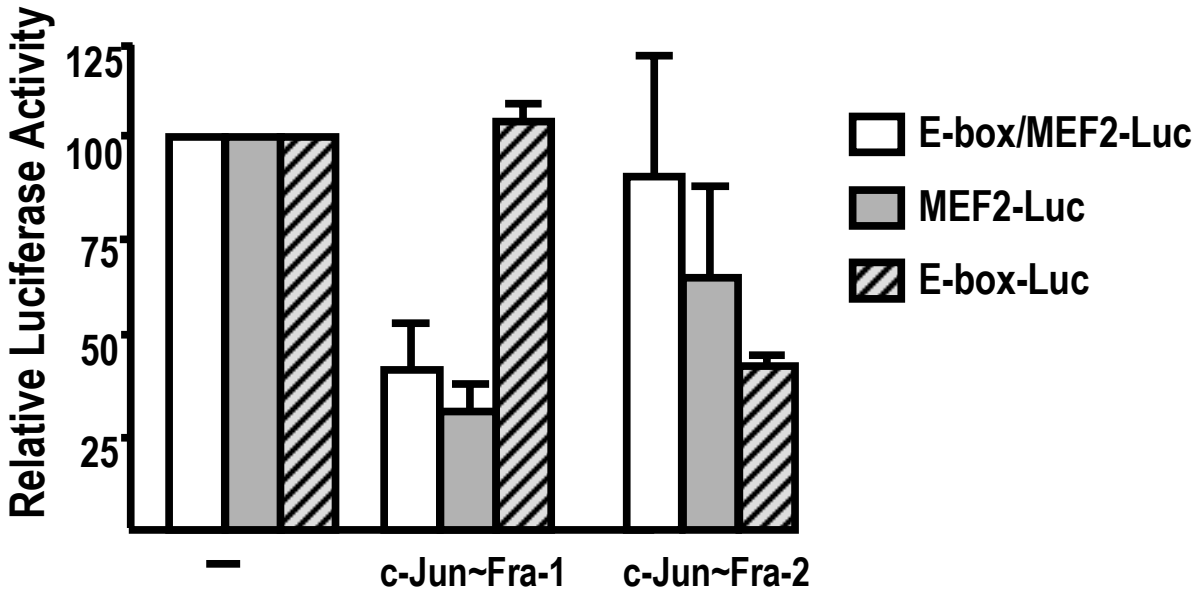

Figure 3.6. cJun~Fra1 and cJun~Fra2 inhibit myogenesis using different mechanisms. 23A2 cells were transiently transfected with either 2.5 μg cJun~Fra-1 or cJun~Fra-2, 50 ng pRL-tk and 1 μg of one of the following reporters: 4Rtk-luc, -228Mgn-luc, or -228mEmgn-luc. Forty-eight hours following induction of differentiation, the cells were lysed and luciferase activities were measured. Cells transfected with vector-only was set to 1.

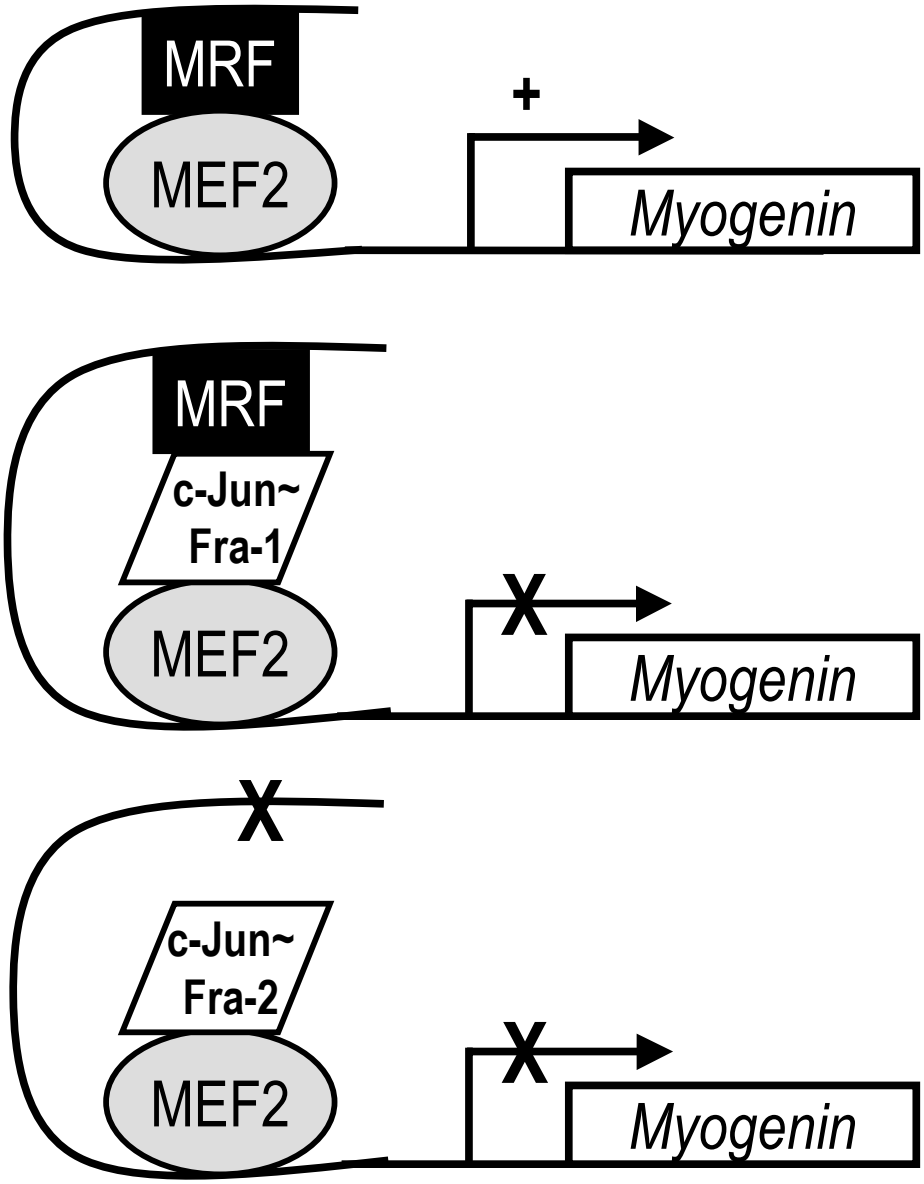

Figure 3.7. Proposed model for AP-1 inhibition of myogenesis. Transcriptional activation of the *myogenin* gene is regulated by the combined actions of the MRFs and MEF2 (upper panel). Upregulation of c-Jun~Fra-1 inhibits the transcriptional activation of *myogenin* by modulating the activity of MEF2 (middle panel). An increase in the amount of c-Jun~Fra-2 inhibits the transcription of myogenin by directly interfering with the E-box within the myogenin promoter, which prohibits MRF binding.

Chapter 4

SUMMARY AND CONCLUSIONS

Summary and Conclusions

The myogenic process is commonly used as a model system to more fully understand the molecular mechanisms underlying developmental problems. Growth factors generally exert their effects by ligand binding to external cellular receptors, activating small intracellular GTPases, and initiating intracellular signaling cascades. Ras is a membrane localized GTPase that is capable of inducing multiple signaling pathways, one of which is the conventional Raf/MEK/MAPK pathway (McCormick, 1995). Constitutively activated forms of Raf kinase induce morphological transformation and are inhibitory to terminal differentiation of skeletal muscle cells in vitro (Dorman and Johnson, 1999). This study was intended to further our understanding of the mechanisms involved in Raf-induced myogenic inhibition.

In order to negatively regulate skeletal myogenesis, Raf-kinase alters the expression of genes that are normally expressed in the myoblast. Therefore, the first study was designed to examine differentially expressed genes in a subtractive screen of Raf-transformed myoblasts. The chicken homologue of TaxREB107 was identified in this screen and characterized. The family of TaxREB proteins consists of six transcriptional regulators that were identified in screens looking for proteins that bind to the enhancer of the human T-cell leukemia virus type I (HTLV-I) long terminal repeat (Yoshimura et al, 1990;Tsujimoto et al, 1991;Morita et al, 1993;Nyunoya et al, 1993). TaxREB107 is a binding protein that has been previously reported to associate with MyoD, and is therefore a potentially important factor during skeletal myogenesis (Riou et

al, 2000). Each of these factors contains the DNA binding and dimerization motif known as the basic leucine zipper (bZip) (Yoshimura et al, 1990;Wagner and Green, 1993;Perini et al, 1995; Baranger et al, 1995). Myogenin, a transcriptional regulator that is required by muscle for terminal differentiation, contains binding elements for proteins containing the bZip motif. Due to the critical role of the MRFs during myogenesis, it is highly plausible that the upregulation of bZip transcription factors by Raf-kinase and their subsequent interaction with the MRFs may contribute to the inhibition of myogenesis by Raf-kinase. Overexpression of this transcription factor modestly increased muscle reporter gene activity. However, it did not alter MyoD-directed transcriptional activity. Immunocytochemistry revealed that the cTaxREB107 protein localized to the nucleus as well as the cytoplasm. This is in agreement with reports that TaxREB107 proteins are ribosomal components, which are transcribed and assembled on the nucleoli within the nucleus. Therefore, TaxREB107 could possibly function at the RNA level to assist with the elevated rate of protein synthesis that occurs in developing skeletal muscle. Taken together, these findings suggest a role for cTaxREB107 in regulation of muscle gene expression during development of skeletal muscle. However, the precise role by which Raf-kinase controls the transcriptional regulation is not presently understood. Because the factor is a positive regulator of myogenesis, it is plausible that Raf-kinase targets the expression of TaxREB107 and the downregulation of this gene contributes to the overall block to mogenesis imposed by Raf.

A stable myogenic cell line containing an inducible form of Raf-kinase was created in order to further investigate the Raf-imposed block to myogenesis. Induction of high levels of Raf-kinase in these cells completely represses the myogenic program. In a microarray experiment to detect differentially expressed gene products, TGFβ_1 was found to be upregulated in Raf-transformed myoblasts three-fold over the parental cell line. Overexpression of TGFβ has been implicated in pathological conditions such as neoplasia because overexpression in vitro promotes proliferation and prevents differentiation in certain cell types, including myoblasts (Florini et al, 1986; Olson et al, 1986; Massague et al, 1986). In fact, two of the three mammalian isoforms of TGFβ, TGFβ_1 and TGFβ_3, may contribute to the development of rhabdomyosarcoma, the most common soft tissue tumor in childhood (McCune et al, 1993). Further analysis of the Raf-overexpressing myoblasts revealed an increase in TGFβ_1 expression and activity. However, blocking the activity of TGFβ_1 failed to reinstate the myogenic program. These findings suggest that although TGFβ_1 may play a role in Raf-induced myogenic inhibition, this growth factor is not solely responsible for the negative effect. However, other members of this superfamily are likely to be involved. One such possible family member is GDF-8, also known as myostatin. This growth factor is expressed primarily in muscle cells throughout development and has been implicated as a strong negative effector in skeletal muscle differentiation (McPherron et al, 1997). Future work is aimed at determining the involvement of this growth factor in Raf-imposed inhibition to myofiber formation.

In the third portion of this study, we chose to further examine the interplay between the Raf-kinase and TGFβ pathways. Raf-kinase has been shown to induce AP-1 activity, while TGFβ primarily utilizes Smad transcriptional complexes in order to exert its effects on myoblasts (Bengal et al, 1992; Dorman and Johnson 2000; Attisano and Wrana, 2002). Although Smad complexes are capable of weakly activating transcription alone, interaction of the activated Smad transcription factor with AP-1 synergistically enhances transcriptional activation (Shi et al, 1998; Liberati et al, 1999). Interestingly, our results indicated that both $TGF\beta_1$ and activated Raf-kinase were capable of increasing AP-1 transcriptional activity. Although AP-1 is generally thought to have a negative effect on muscle differentiation, the cellular effects of this transcriptional regulator are diverse (Angel et al, 1991). The varied cellular responses to AP-1 most likely depend on the individual components of AP-1, which include Jun and Fos (Allegretto et al, 1990; Halazonetis et al, 1988; Nakabeppu et al, 1988; Smeal et al, 1989; Ransone et al, 1990). For instance, fibroblasts that have been transformed by Ras have increased levels of c-Jun, while levels of JunD are diminished (Pfarr et al, 1994). The precise role of AP-1 in skeletal myogenesis is not well defined. Previous studies have indicated that c-Jun directly associates with myogenin and MyoD, which results in myogenic inhibition (Bengal et al, 1992; Li et al, 1992).

In order to determine which components of AP-1 are present in myoblasts inhibited by Raf-kinase or excess amounts of $TGF\beta_1$, we examined the expression and activity of each AP-1 subunit. In both cases, the presence of c-

Jun, Fra-1 and Fra-2 was observed. Overexpression of tethered AP-1 dimers in myoblasts revealed that both c-Jun~Fra-1 and c-Jun~Fra-2 tethers inhibit skeletal myogenesis. Interestingly, further examination of the mechanisms used by each of these tether proteins suggested that each uses a different mode of action in order to exert its effects. c-Jun~Fra-1 appears to target MEF-2, while c-Jun~Fra-2 requires the presence of an E-box in order to negatively impact skeletal muscle formation. These findings suggest that the individual components of AP-1 determine cellular responses to the transcriptional regulator. Further work detailing whether AP-1 components are directly binding to MEF-2 or the MRFs would give a clearer view to the precise mechanisms involved in the block to myogenesis imposed by these transcriptional regulators.

Inhibition of skeletal myofiber formation by Raf most likely involves the use of several different intracellular mechanisms. A proposed model for this is shown in Figure 5.1. When the Raf/MEK/ERK pathway is activated, expression of a TGFβ family member, possibly GDF-8, is upregulated and allows the growth factor to work in an autocrine manner on the cell. Additionally, activated Raf-kinase increases the phosphorylation status of c-Jun. An active AP-1 transcriptional complex is created when c-Jun partners with either Fra-1, whose expression is increased by both Raf and TGFβ, or Fra-2. c-Jun:Fra-1 targets MEF2 transcriptional activation possibly by acting in a dominant negative manner. Conversely, myogenic regulatory factor transcriptional activity is inhibited by c-Jun:Fra-2 dimer formation. A third possible mechanism that may

be downregulated in response to Raf-kinase is TaxREB107, which plays a positive role during skeletal myogenesis.

The overall goal of this work was to further elucidate the precise mechanisms used by activated Raf-kinase in order to block myogenesis. Although a single pathway was not found to be responsible for these effects, the complexities of the intracellular processes are evident. The phenomenon of prevention of skeletal muscle fiber formation by Raf-kinase requires the employment of many different processes and components within the cell. A completed picture of the mechanisms involved will greatly advance identification of targets for treatment of certain types of cancer. Future work will be required to complete our understanding of the pathways involved in myogenic inhibition imposed by activated Raf and to answer several intriguing questions. Chief amongst these is to uncover the link between the Raf-kinase and TGFβ signaling pathways. Rhabdomyosarcoma is a common soft tissue tumor that is diagnosed in four to seven million children younger than age 15 each year (Young et al, 1986). Inappropriate expression of TGFβ plays a large role in the carcinogenesis of these tumors (McCune et al, 1993). Currently, the treatment strategies available for patients with cancerous conditions such as this are limited to surgery, radiation, and chemotherapy. While somewhat effective, these courses of treatment are extremely toxic and often cause other complications (Dagher and Helman, 1999). Because the steps involved in intracellular signaling pathways are altered in human disease states, each one is a potential target for therapeutic approaches to the neoplastic condition. The use of neutralizing

antibodies to target excessive autocrine growth factors and gene therapy to introduce dominant negative components of altered signaling pathways are both plausible approaches to the treatment of cancerous conditions.

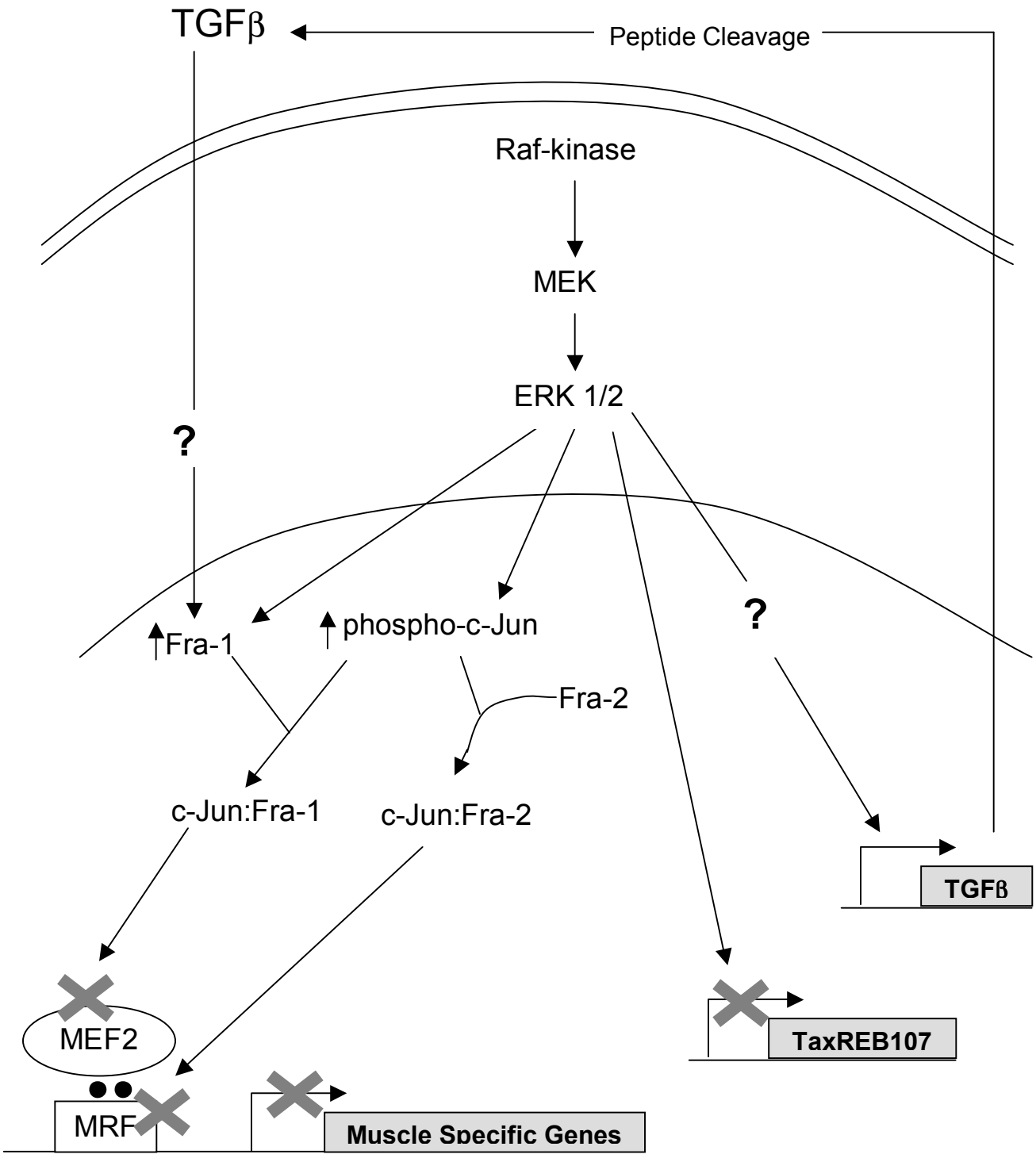

Figure 5.1. Proposed model of the role of AP-1 in the Raf-imposed block to skeletal myogenesis.

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APPENDIX A: ABBREVIATIONS

AP-1, activator protein 1

ATF, activating transcription factor

bHLH, basic helix-loop-helix

bZip, basic leucine zipper

CREB, cyclic AMP response element binding

ERK1/2, extracellular signal-related kinase 1 and 2

FGF, Fibroblast growth factor

GDF, growth and differentiation factor

HDAC, histone deacetylase

IGF, insulin-like growth factor

JNK, c-jun-N-terminal kinase

MADS, minichromosome maintenance 1 (MCM1), Agamous, Deficiens and serum response factor (SRF)

MAPK, mitogen-activated protein kinase

MEF2, myocyte enhancer factor 2

MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase

MRF, myogenic regulatory factor

PI3-kinase, phosphatidylinositol 3-kinase

RACE, rapid amplification of cDNA ends

SAPK, stress-activated protein kinase

SARA, Smad anchor for receptor activation

TAD, transcriptional activation domain

TAK1, TGFβ-activated kinase 1

TaxREB107, tax responsive element binding 107

TGF-β, transforming growth factor beta

TPA, 12-O-tetradecanoyl phorbol-13-acetate

TRE, TPA response element

Appendix B

TRANSFORMING GROWTH FACTOR BETA-1 (TGF-β_1) IS UP-REGULATED BY ACTIVATED RAF IN SKELETAL MYOBLASTS BUT DOES NOT CONTRIBUTE TO THE DIFFERENTIATION-DEFECTIVE PHENOTYPE

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I. Abstract

The Raf/MEK/MAPK signaling module elicits a strong negative impact on skeletal myogenesis that is reflected by a complete loss of muscle gene transcription and differentiation in multinucleated myocytes. Recent evidence indicates that Raf signaling also may contribute to myoblast cell cycle exit and cytoprotection. To further define the mechanisms by which Raf participates in cellular responses, a stable line of myoblasts expressing an estrogen receptor-Raf chimeric protein was created. The cells (23A2RafER^{DD}) demonstrate a strict concentration dependent increase in chimeric Raf protein synthesis and downstream phosphoMAPK activation. Initiation of low-level Raf activity in these cells augments contractile protein expression and myocyte fusion. By contrast, induction of high-level Raf activity in 23A2RafER^{DD} myoblasts inhibits the formation of myocytes and muscle reporter gene expression. Interestingly, treatment of myoblasts with conditioned medium isolated from Raf-repressive cells inhibits all aspects of myogenesis. Closer examination indicates that the TGF-β_1 gene is up-regulated in Raf-repressive myoblasts. The cells also direct elevated levels of Smad transcriptional activity suggesting the existence of a TGF-β_1 autocrine loop. However, extinguishing the biological activity of TGF-$\beta_1$does not restore the myogenic program. Our results provide evidence for the involvement of Raf signal transmission during myocyte formation as well as during inhibition of myogenesis.

II. Introduction

Myogenesis, the formation of contractile-competent skeletal muscle cells, is tightly regulated by the presence of several critical growth factors in the extracellular environment. As an example, local production of insulin-like growth factor-I is a strong permissive growth factor for both morphological and biochemical differentiation of myoblasts (1-4). Ectopic IGF-I delivery to skeletal muscle cells increases muscle mass and morphometric measures of strength in birds and mice (5;6). On the contrary, several laboratories have documented the repressive effects of transforming growth factor beta-1 ($TGF\beta_1$) and members of the fibroblast growth factor family, chiefly FGF2 (for review see (7;8)). The mechanism by which these soluble inhibitory proteins impede skeletal myogenesis involves the activation of specific intracellular signaling cascades. In the case of $TGF\beta_1$, a requisite induction of Smad3 protein phosphorylation and nuclear translocation occurs that interferes with muscle gene transcription (9). The precise mechanism for FGF2 mediated inhibition of myogenesis is less understood but may involve downstream signaling events that are controlled through G-proteins (10).

Overexpression studies and application of specific kinase inhibitors has led to the identification of several essential signaling pathways in skeletal myoblasts. It is firmly established that phosphatidylinositol 3-kinase (PI3K) and p38, a stress-activated mitogen activated protein kinase (MAPK), both are necessary for the formation of mature myocytes (11-15). Removal of downstream signaling events by the respective kinases through the use of

chemical inhibitors abolishes myogenesis. By contrast, initiation of downstream signaling modules by constitutively active Ras, a membrane localized GTPase that participates in numerous receptor tyrosine kinase initiated signaling events, results in a severe reduction in muscle gene transcription and myoblast fusion (16;17). Activation of Raf, the downstream target of Ras, also abolishes the myogenic program (18-21). Interestingly, the means by which Ras and Raf inhibit muscle gene expression does not appear to be directly dependent upon the subsequent phosphorylation and activation of extracellular regulated kinases 1 and 2 (ERK1/2). Inhibition of MEK/ERK signal transduction does not reinstate the full complement of muscle gene transcription and myoblast fusion to myoblasts expressing constitutively active Ras or Raf (17;22).

Raf kinase is a serine/threonine kinase whose activation precludes ERK1/2 (MAPK) phosphorylation and a concomitant alteration in gene transcription (23;24). Signaling through the Raf/MEK/ERK pathway is a common mitogenic response for many cell types. Recently, several groups have reported differential cellular responses as a consequence of Raf/MAPK signal intensity. At lower levels of MAPK activity, epithelial and fibroblasts demonstrate increased proliferative rates (25;26). By contrast, high levels of Raf/MAPK result in cell cycle arrest and senescence (26;27). The Raf-mediated responses are attributed to signaling through MEK/MAPK as well as non-MEK dependent signaling events. Regulation of Raf activity indicates that the protein physically associates with other kinases, regulatory proteins and scaffolding proteins [for review see (28)]. Indeed, it has become increasingly apparent that Raf kinase

can control cellular transcriptional responses through mechanisms that are independent of the archetypical MEK/ERK module. A Raf kinase allele that fails to interact with MEK and cause ERK1/2 phosphorylation retained the ability to activate NFκB-directed transcription and promote neuronal differentiation (29). These results argue that Raf participates in several downstream signaling cascades. Differential utilization of these pathways may be reflected in the cell's decision to undergo proliferation, differentiation, apoptosis and senescence.

Because many growth factors exhibit contrasting effects on skeletal myogenesis yet utilize many of the same intracellular signaling pathways, it is likely that signal intensity plays a critical role in the decision to complete terminal differentiation. To this end, myoblasts that express an inducible activated Raf allele were created. Our results indicate that low-level Raf activity promotes myogenesis while high-level Raf activity inhibits muscle formation. Coincident with repression of differentiation by activated Raf is an increase in TGFβ_1 gene expression and Smad-directed transcriptional activity. However, removal of TGFβ_1 from the extracellular environment of the Raf-repressive myoblasts does not reinstate the differentiation program. In summary, Raf signaling modules are both positive and negative mediators of myogenesis that is a direct reflection of signal strength.

III. Materials And Methods

Cell culture, plasmids and transfections

23A2RafER^{DD} myoblasts were created by transduction of 23A2 myoblasts with a retrovirus encoding the fusion protein RafER^{DD} (30). RafER^{DD} is comprised of the kinase domain of human c-Raf-1 fused in frame with the estrogen receptor ligand binding domain. Following infection, the cells were selected in puromycin and clones were isolated by limiting dilution. 23A2 and 23A2RafER^{DD} myoblasts were cultured on gelatinized tissue culture grade plasticware in Dulbecco's modified Eagle medium (DMEM) containing 15% fetal bovine serum (BioWhittaker), 1% penicillin-streptomycin and 0.5% gentamicin (Invitrogen, Carlsbad, CA). Differentiation was induced in confluent cultures by continuous culture in DMEM supplemented with 2% horse serum, 1% penicillin-streptomycin and 0.5% gentamicin. For the measurement of muscle-specific reporter gene activity, myogenic cells cultured in 6-well tissue plasticware were transiently transfected with 1 μg of troponin I luciferase (TnI-Luc), the minimal E-box reporter plasmid, 4Rtk-luciferase (4Rtk-Luc) or a multimerized AP-1 binding site reporter (AP1-Luc) and 50 ng of pRL-tk, a *Renilla* luciferase plasmid as a monitor of transfection efficiency, using standard calcium phosphate methods (16;22). The cells were maintained in differentiation media for 48 hours in the presence or absence of varying concentrations of 4-hydroxy-tamoxifen (4HT;Sigma, St. Louis, MO) prior to lysis and measurement of luciferase activities (Dual-luciferase, Promega, Madison, WI). The amount of corrected luciferase activity generated by 23A2 or 23A2RafER^{DD} myoblasts treated with vehicle was set to 100%. Each experiment was replicated a minimum of three times. For conditioned medium experiments, the culture medium was removed

from myoblasts treated for 48 hours with DMSO or varying amounts of 4HT. The media was centrifuged to remove debris and brought to a final concentration of 2% horse serum. The resulting medium was applied to confluent 23A2 myoblasts and the cells were maintained for an additional 48 hours prior to fixation.

RNA isolation, Northern blots and RT-PCR

Total RNA was isolated with STAT60 denaturing solution per the manufacturers recommendations (Tel-Test, Friendswood, TX). Twenty micrograms of total RNA were separated through 1% agarose gels containing 2% formaldehyde, transferred to nitrocellulose and irreversibly cross-linked to the medium by UV. The blots were hybridized with ^{32}P-labeled cDNA probes generated by random hexamer priming (DecaPrime, Ambion, Houston, TX). The probes corresponded to regions contained within the TGF-β_1 and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene coding regions. Northern blot analysis was performed using Ultra-Hyb (Ambion, Houston, TX) at 42 C overnight according to the manufacturers recommendations. Blots were washed with 2XSSC/0.1% SDS once at room temperature and twice with 1XSSC/0.1% SDS at 42 C. Blots were exposed to phosphorimaging screens for the visualization and quantification of message levels. For the semi-quantitative assessment of mRNA levels, 1 μg of total RNA was reverse transcribed with MMLV reverse transcriptase. Amplification was performed using Taq polymerase (Fisher Scientific, Pittsburgh, PA), gene-specific primers and

thermocycle conditions of 95 C for 45 seconds, 55 C for 45 seconds, 72 C for 90 seconds for a total of 35 cycles. To ensure that quantification was accomplished in the linear range of amplification, an aliquot was removed from each reaction after 25, 30 and 35 cycles. Amplicons were separated through 2% agarose gels containing ethidium bromide, visualized under UV and photographed.

Western blot and scanning densitometry

23A2RafER^{DD} myoblasts were differentiated in the presence or absence of 4HT. After 48 hours, the cells were lysed in 4X SDS-PAGE sample buffer and an aliquot removed for protein quantification. Equal amounts of protein were electrophoretically separated through denaturing gels and transferred to nitrocellulose. The blots were incubated with 5% nonfat dry milk in TBST (10 mM TRIS, pH 8.0, 150 mM NaCl, 0.1% Tween-20) to block nonspecific binding sites. Primary antibodies were diluted in blocking buffer and the blots were incubated overnight at 4 C with shaking. Antibodies and dilutions included: anti-myogenin (F5D ascites, Developmental Hybridoma Bank, University of Iowa, Iowa; 1:5,000), anti-myosin heavy chain (MF20 hybridoma supernatant, Developmental Hybridoma Bank, University of Iowa, Iowa; 1:5), anti-ERK1/2 and anti-phosphoERK1/2 (Cell Signaling), anti-estrogen receptor (Santa Cruz Biotech, Santa Cruz, CA; 1:300). After extensive washing with TBST, the blots were reacted with the appropriate peroxidase secondary antibody for 45 minutes at room temperature. Visualization of protein bands was accomplished by chemiluminescence and autoradiography. Multiple exposures to X-ray film were

used to ensure that the linear range of densitometry was maintained. Audoradiograms were scanned on a Storm 860 phosphorimaging system (Molecular Dynamics, Amersham Biosciences, Piscataway, NJ).

Immunocytochemistry

23A2 and 23A2RafER^{DD} myocytes were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes at room temperature. The cells were washed with PBS and nonspecific antigen sites blocked by incubation with 5% horse serum in PBS containing 0.1% Triton X100 for 20 minutes at room temperature. Cultures were incubated with anti-myosin heavy chain (MF20) for 60 minutes at room temperature. After exhaustive washing with PBS, the cells were reacted with donkey anti-mouse FITC (Vector Labs, Burlingame, CA; 1:200) for 45 minutes at room temperature. Cultures were washed with PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI). Immunofluorescent detection was accomplished using a Nikon TE200 inverted phase microscope equipped with epifluorescence. Representative photomicrographs were captured to slide film and assembled in Adobe Photoshop.

IV. Results

Creation and characterization of an inducible Raf myogenic cell line.

Previous work has clearly demonstrated that overexpression of activated alleles of Raf are inhibitory to myocyte formation and muscle gene expression (17;18;21). More importantly, MAPK-dependent repression appears to be a function of Raf signaling intensity in avian myoblasts (18). To extend these observations, an activated Raf kinase that is responsive to the estrogen analog, 4-hydroxy tamoxifen (4HT), was stably expressed in 23A2 mouse myoblasts (23A2RafER^{DD}). Confluent cultures of 23A2RafER^{DD} myoblasts were differentiated in the presence or absence of increasing concentrations of 4HT. After 48 hours, the cells were lysed for Western analysis of chimeric Raf (α-estrogen receptor), activated MAPK (α-phosphoMAPK) and total MAPK (α-MAPK) expression. As shown in Figure 1, a dose-dependent increase in the relative amount of RafER^{DD} protein is found with amounts 4HT from 0.25 nM to 2.5 μM (lanes 2-6). Coincident with the increase in chimeric Raf protein is an elevation in the amounts of phosphorylated MAPK. Phosphorylated ERK2 is preferentially activated at 4HT concentrations as low as 0.25 nM while activated ERK1 is first apparent at 4HT concentrations of 25 nM or greater. No differences in the amounts of total MAPK (ERK1/2) were evident.

Activation of ERK1/2 signal transmission leads to an increase in AP-1 directed transcriptional activity in many cell types, including skeletal myoblasts. To ensure that the Raf/ERK pathway was functional in 23A2RafER^{DD} myoblasts, semiconfluent cells were transiently transfected with a multimerized AP-1

reporter plasmid and pRL-tk, a plasmid encoding Renilla luciferase as a marker of transfection efficiency. Subsequently, the cells were treated with increasing amounts of 4HT for 48 hours prior to lysis and measurement of luciferase activities. AP-1 Luc reporter activity was normalized to Renilla luciferase activity. Results demonstrate a dose-dependent increase in AP-1 Luc activity with increasing amounts of 4HT (Figure 2). At concentrations of 4HT greater than 250 nM, a decline in AP-1 luciferase activity was evident. These results indicate that the Raf/ERK signaling axis is intact and functional in the myogenic cells.

As final confirmation of the integrity of the Raf signaling system in 23A2RafER^{DD} myoblasts, confluent cultures of cells were treated for 48 hours in differentiation medium containing vehicle-only (DMSO) or 1 μM 4HT. Subsequently, cells were fixed and immunostained for myosin heavy chain (α-MyHC). Control 23A2RafER^{DD} myocytes treated with vehicle readily differentiate into large multinucleated myofibers that express copious amounts of the contractile protein, MyHC (Figure 3). By contrast, activation of the Raf/ERK signaling pathway leads to a severe reduction in both myofiber number and MyHC protein expression. These results directly reflect published reports of the effects of activated Raf on skeletal myogenesis. Thus, 23A2RafER^{DD} myoblasts represent a myogenic cell line that retains its muscle features in the absence of Raf activity and is differentiation-defective in the presence of Raf signal transmission.

Contrasting effects of Raf signal transduction on skeletal myoblasts.

To understand the effects of signal intensity of myogenesis, 23A2RafER^{DD} myoblasts were differentiated in the presence of increasing amount of 4HT. After 48 hours, the cells were lysed and equal amounts of total cellular proteins were analyzed by Western blot for the expression of muscle proteins (anti-MyHC, anti-myogenin). Scanning densitometry was performed on the chemiluminescent autoradiograms. Muscle protein expression was normalized to total ERK2 expression, an internal marker for protein loading, transfer and detection efficiency. In the absence of 4HT, 23A2RafER^{DD} myoblasts differentiate into myocytes that express both MyHC and myogenin (Figure 4). Unexpectedly, low-level induction of Raf activity using concentrations of 4HT (0.25 or 2.5 nM) causes an increase in the amount of contractile and regulatory protein expression. Supplementation of differentiation medium with 25 nM 4HT or greater causes the predicted loss of MyHC and myogenin protein expression. The loss of muscle marker protein expression is not a consequence of 4HT as the estrogen analog did not significantly alter the ability of 23A2 myoblasts to differentiate (data not shown). The biphasic response of 23A2RafER^{DD} myoblasts to increasing 4HT concentrations is further reflected in muscle-specific reporter gene activity (Figure 5). 23A2RafER^{DD} myoblasts were transiently transfected with troponin-I luciferase (TnI-Luc) and pRL-tk. After 48 hours in differentiation medium, the cells were lysed and luciferase activities measured. The levels of Raf activity directed in response to 25 nM 4HT or greater are sufficient for a reduction in TnI-Luc activity. However, low-level Raf activity (0.25-

2.5 nM 4HT) directs only a slight increase in muscle reporter gene activity. It is likely that a larger portion of the TnI regulatory region is necessary to obtain a substantial increase in reporter gene transcription. Our results clearly demonstrate that Raf has contrasting effects on muscle formation that are intensity dependent.

Raf inhibition of myogenesis involves secretion of a soluble factor.

It has been reported that the inability of myoblasts to differentiate in the presence of activated Raf is a product of sequestration of MEF2 in the cytoplasm (21). However, forced expression of the transcription factor in avian myoblasts does not reinstate the muscle gene program arguing that additional factors are involved (31). Moreover, rhabdomyosarcoma cells secrete bioactive TGF-β_1 that acts in an autocrine manner to suppress differentiation (32). To further characterize the differentiation-defective phenotype of myoblasts directing extreme levels of Raf/ERK signal, conditioned medium was collected from 23A2RafER^{DD} myoblasts treated with DMSO, 2.5 nM or 2.5 μM 4HT in differentiation medium. Cell debris was removed by centrifugation and the supernatants were supplemented with horse serum to a final concentration of 2%. Confluent cultures of 23A2 myoblasts were allowed to differentiate for 48 hours in the conditioned medium followed by fixation and immunostaining for MyHC. Control cultures were treated with DMSO, 2.5 nM or 2.5 μM 4HT and analyzed as described. Conditioned medium from 23A2RafER^{DD} myoblasts differentiated in the presence or absence of 2.5 nM 4HT did not alter the ability of

myoblasts to fuse into MyHC positive myofibers (Figure 6). By contrast, medium harvested from 23A2RafER^{DD} myoblasts treated with 2.5 μM 4HT dramatically inhibited myocyte formation. Less than 1% of the myoblasts were able to fuse into myosin expressing myofibers. Control myoblasts were not affected by 2.5 μM 4HT to the same extent. The complete absence of differentiated cells strongly argues that a soluble factor is secreted from Raf-expressing myoblasts that contributes to the loss of myofiber formation.

TGFβ_1 does not contribute to the Raf imposed block to myogenesis.

Differentiation defective rhabdomyosarcoma cells secrete TGFβ_1 into the culture medium that contributes to the loss of myogenic potential. To determine if a TGFβ-like factor may be responsible for the inhibition of muscle formation in myoblasts treated with Raf-repressive conditioned medium, 23A2 myoblasts were transiently transfected with 3TP-Lux, a reporter plasmid whose activity is modulated by TGFβ proteins, and pRL-tk. Cells were treated for 48 hours in conditioned medium harvested from 23A2RafER^{DD} myoblasts treated with DMSO, 2.5 nM or 2.5 μM 4HT. Subsequently, the cells were lysed and luciferase activities measured. Myoblasts that are capable of fusing and expressing contractile proteins (DMSO, 2.5 nM 4HT) do not direct a significant amount of 3TP-Lux activity (Figure 7). However, myoblasts that are unable to express the differentiated phenotype demonstrate a six-fold increase in TGFβ responsive reporter gene function. Therefore, we conclude that a TGFβ-like factor is present

in the medium harvested from 23A2RafER^{DD} myoblasts transmitting high-level Raf signals.

To determine if the soluble factor is TGFβ_1, gene specific primers for the growth factor were designed for the amplification of messages by RT-PCR. In brief, total RNA was harvested from 23A2 and 23A2RafER^{DD} myoblasts treated for 48 hours in differentiation supplemented with 2.5 μM 4HT. Equal amounts of RNA were reversed transcribed and amplified using primers specific for the TGFβ_1 and GAPDH. The cDNAs were amplified with Taq polymerase and an aliquot of each reaction was removed after 25, 30 and 35 cycles (see Materials and Methods for conditions). Reaction products were separated through 2% agarose gels containing ethidium bromide. By semi-quantitative PCR, a cycle-dependent increase in GAPDH was evident in both control and Raf-repressive samples (Figure 8A). No amplicons for TGFβ_1 were detected in RNA isolated from control myocytes but gene products for the growth factor were evident after 30 and 35 cycles in 23A2RafER^{DD} myoblasts treated with 4HT (Raf-repressive) RNA samples. Northern blot analysis confirmed the increase in TGFβ_1 mRNA in Raf expressing myoblasts (Figure 8B).

Our results indicate that TGFβ_1, a potent inhibitor of muscle differentiation, is produced by 23A2RafER^{DD} myoblasts exhibiting extreme levels of Raf signaling and propose that this protein may be responsible for the block to muscle formation. Therefore, removal of secreted TGFβ may reverse the inhibition of myogenesis in myoblasts with high-level Raf signal intensity. To this end, cultures of 23A2RafER^{DD} myoblasts treated with DMSO or 500 nM 4HT

were incubated for 48 hours in differentiation medium supplemented with a soluble inhibitor of TGFβ. The TGFβ inhibitor (βSRI) is a chimera of the TGFβ receptor ligand binding domain fused to human Fc portion of IgG that prevents TGFβ_1, -β_2 and -β_3 from interacting with cell surface receptors (33;34). After 48 hours, the cells were fixed and immunostained for MyHC and numbers of immunopositive cells were measured. 23A2RafER^{DD} myoblasts in the absence of 4HT readily fuse into large multinucleated, MyHC immunopositive fibers (Figure 9). Treatment of the cells with TGFβ_1 (2 ng/ml) suppressed myocyte formation to less than 25% of control indicating that the cells respond to the growth factor in a manner analogous to parental 23A2 skeletal myocytes. This level of inhibition also is attained by treatment of 23A2RafER^{DD} myoblasts with 500 nM 4HT. However, supplementation of the culture medium with βSRI at a concentration that effectively restores myogenesis to TGFβ_1 inhibited myoblasts does not reinstate the differentiation program to 23A2RafER^{DD} myoblasts directing elevated Raf signaling. Thus, we conclude that TGFβ_1 inhibits the differentiation of 23A2RafER^{DD} myoblasts, that the cells produce TGFβ_1 in response to elevated Raf but the establishment of an autorcrine TGFβ_1 loop in these cells does not significantly contribute to the differentiation-defective phenotype imposed Raf signaling.

V. Discussion

Activated Raf signal transmission represents a powerful disruptor of normal myocyte formation and muscle gene expression. The exact mechanism by which activated Raf alleles accomplish this feat remains largely unknown. Several contributing factors to the loss of myogenic capacity include inhibition of myogenin gene expression and cytoplasmic sequestration of MEF2 protein (21;31). Recently, we have demonstrated that forms of activated Raf that initiate activation of differential downstream signaling intermediates cause varying responses on myogenin gene expression that may be correlated to overall signal strength (31). 23A2RafER^{DD} myoblasts were created to provide additional insight into the effects of Raf signal intensity on myocyte formation. In the absence of Raf activity, the cells readily form muscle in a manner analogous to parental 23A2 myoblasts. Interestingly, introduction of a low-level Raf-mediated signal stimulates an increase in the numbers of myocytes formed and enhances their ability to transcribe muscle genes. This is an intriguing finding in light of the fact that medium and high-level Raf activities lead to a reduction in markers of muscle differentiation. Using stable clones of mouse myoblasts that direct differing levels of Raf kinase activity, DeChant reported suboptimal Raf signal transmission leads to an increase in muscle reporter gene transcription and myocyte formation (35). The increase in myogenic capacity is not a reflection of diminished apoptosis as the low-level Raf clone and parental myoblasts did not differ in the extent of DNA fragmentation. Therefore, it is likely that enhanced muscle formation in response to nominal Raf-mediated signaling occurs

independent of enhanced cell survival. Others have suggested that Raf inhibits myogenesis by maintaining the cell in a proliferative state (20). L6 myoblasts expressing an estrogen-inducible allele of activated Raf fail to express muscle genes or fuse into myocytes upon initiation of Raf signaling. In addition, these cells demonstrate an increased mitotic index in response to the kinase. 23A2RafER^{DD} myoblasts and primary avian myoblasts expressing activated Raf are refractile to the mitogenic effects of Raf/MAPK [data not shown, (18)]. These contrasting results may reflect differences in the absolute amounts of Raf signaling.

High-intensity Raf signaling causes cell cycle arrest and senescence in epithelial and fibroblast cells (25;27;30;36). Coincident with mitotic arrest, Raf initiates an increase in TGF-β_1 gene expression leading to creation of an autocrine loop that directly participates in the growth inhibition (37). Similarly, *TGFβ_1* is up-regulated in rhabdomyosarcoma cells and may play a role in the block to myogenic differentiation (32). Our results indicate that elevated Raf signaling does elicit an increase in TGFβ_1 gene expression however, this negative regulator of myogenesis does not contribute significantly to the differentiation-defective phenotype of Raf-expressing myoblasts. 23A2RafER^{DD} myoblasts are inhibited by TGFβ_1 to the same extent as parental cells arguing that the Raf-expressing cells are not refractile to the inhibitory actions of the growth factor and that the cells synthesize a functional TGFβ receptor. It is possible that TGFβ is secreted and retained in the extracellular matrix of the muscle cells thus, leaving it unavailable for sequestration by the soluble TGFβ

inhibitor. This scenario is doubtful as retention of the TGFβ_1 by the extracellular matrix would inactivate the growth factor and would lend itself to promotion of myogenesis, a condition contradictory to those observed. Thus, it is most likely that TGFβ_1 production in response to activated Raf does not play a significant role in suppression of muscle formation.

Treatment of parental myoblasts with conditioned medium from Raf-repressive myoblasts causes a significant increase in the amount of Smad-driven reporter gene transcription. This finding endorses the synthesis and release of a TGFβ-like soluble factor by Raf-expressing cells. The identity of the factor remains unknown. Of the TGFβsuperfamily members, GDF-8 (also referred to as myostatin) represents a strong candidate for the putative secreted factor. GDF-8 myoblast differentiation in vitro and animals lacking or carrying a dysfunctional GDF-8 gene exhibit myocyte hypertrophy (38-42). The growth factor signals through the activin receptor, a membrane-bound receptor that transmits information through the Smad proteins (43;44). Previously, our group reported an inhibition of DNA synthesis in avian myoblasts transduced with a highly active allele of Raf (18). GDF-8 not only inhibits myogenesis but, the growth factor also causes a noticeable reduction in proliferation rates in myoblasts (45;46). These two pieces of information lend support to the notion that GDF-8 acts through an autocrine loop to synergize with Raf signaling and more effectively inhibit myogenesis. However, 23A2RafER^{DD} myoblasts treated with 1 μM 4HT fail to display a mitotic index that differs from control myoblasts (data not shown). As such, it also is possible that a novel TGFβ family member contributes to the

Raf-induced block to muscle formation. In a preliminary microarray analysis of global gene expression in Raf-repressive myoblasts, several GDF genes were up-regulated, including GDF-8 (Wang and Johnson, unpublished results). Future work will examine the function of myostatin in myoblasts directed high levels of Raf/MAPK activity.

In summary, Raf signal transmission directs contrasting effects on skeletal myoblasts that is commensurate with intensity level. Low-level Raf/MAPK signaling elicits a positive effect on myogenesis that is reflected by enhanced regulatory and contractile protein synthesis. By contrast, sustained and extreme levels of Raf signaling leads to the prototypical reduction in myocyte formation and muscle protein expression. Coincident with repression of myogenesis by activated Raf is an increased expression of TGFβ_1 and release of a soluble TGFβ-like factor into the medium. While the identity of the secreted protein remains unknown, it likely is not TGFβ_1 as incubation of Raf-repressive myoblasts with an inhibitor of the growth factor does not reinstate the differentiation program.

ACKNOWLEDGEMENTS
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VI. References

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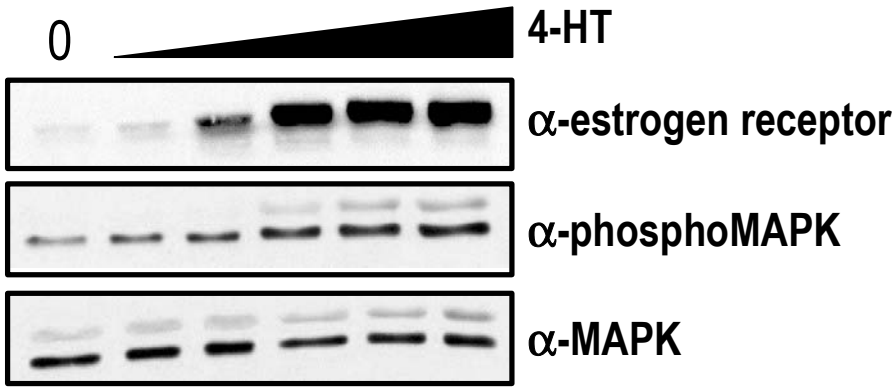

Figure 3.1. Induction of Raf expression produces an increase in ERK1/2 activity in 23A2 myoblasts. 23A2RafER^{DD} myoblasts were cultured for 48 hours in differentiation permissive medium supplemented with 0, 0.25, 2.5, 25, 250 or 2500 nM 4HT. Total cell lystes were analyzed by Western blot for Raf (anti-estrogen receptor), phosphorylated ERK1/2 and total ERK1/2. A dose-dependent increase in chimeric Raf and phosphorylated ERK1/2 is evident.

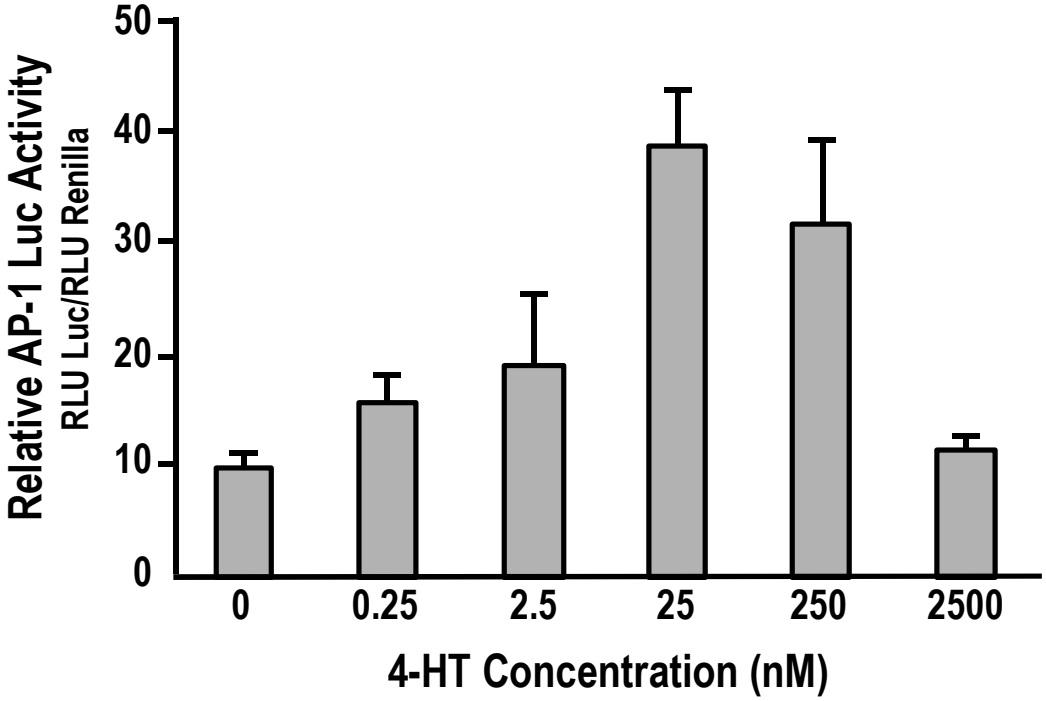

Figure 3.2. *Increasing Raf activity is correlated with an increase in AP-1 directed transcription.* 23A2RafER^{DD} myoblasts (1X10^5) were transiently transfected with 2 mg of AP-1-Luc and 50 ng of pRL-TK. Cells were cultured for 48 hours in differentiation medium supplemented with increasing concentrations of 4HT prior to lysis. AP-1 luciferase activity was normalized to the amount of *Renilla* luciferase activity. Data represents the mean and SEM of at least three independent experiments.

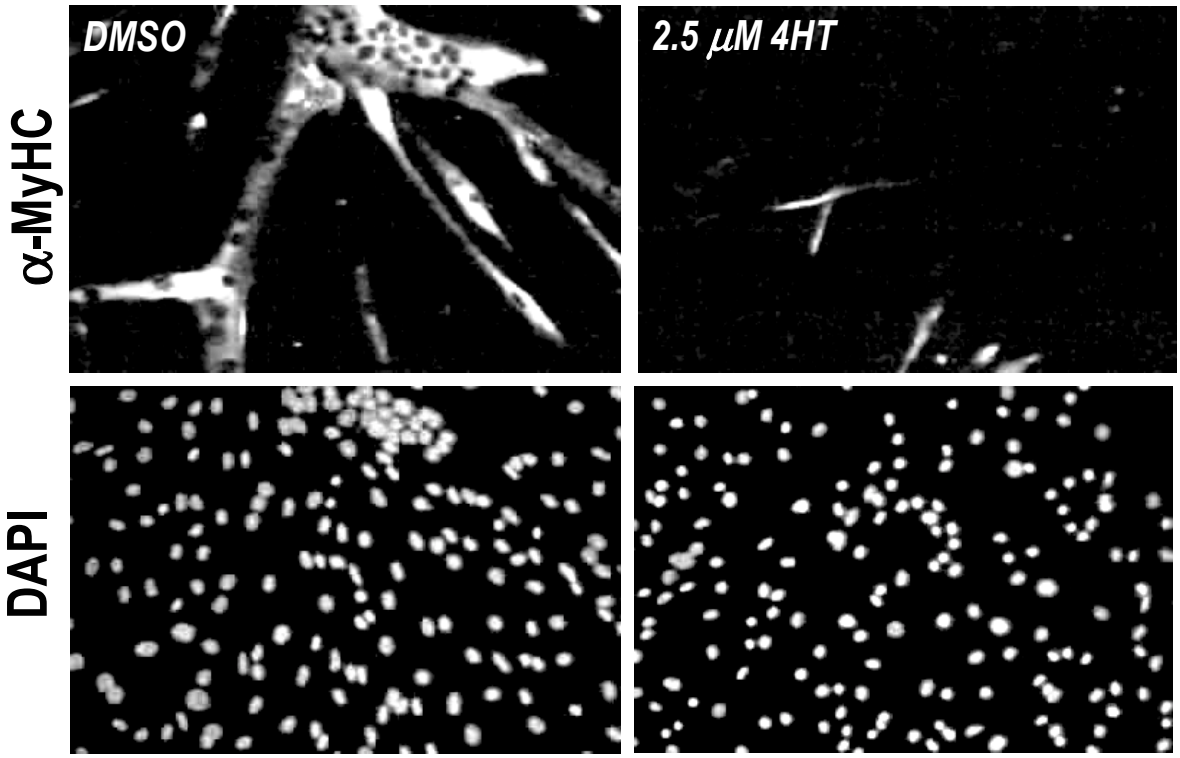

Figure 3.3. *Elevated RafER^{DD} expression inhibits myogenesis.* 23A2RafER^{DD} myoblasts were cultured for 48 hours in differentiation permissive medium supplemented with DMSO or 2.5 μM 4HT. Cells were fixed and immunostained for MyHC expression. Nuclei were visualized by DAPI counterstain. Representative photomicrographs were captured at 400X.

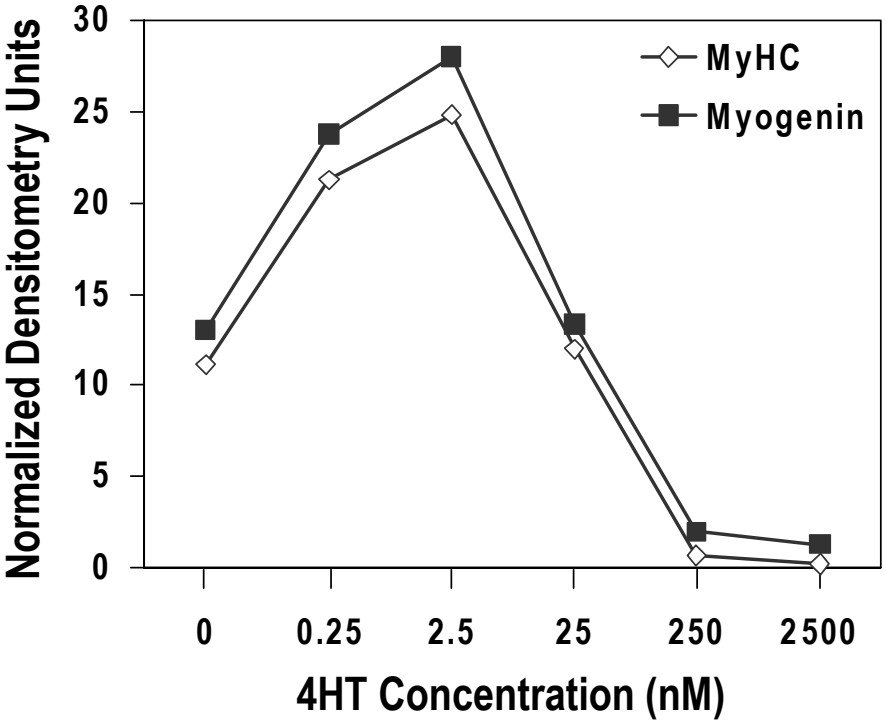

Figure 3.4. *Low-level Raf activity enhances skeletal myogenesis.* 23A2RafER^{DD} myoblasts were cultured in differentiation medium supplemented with increasing amounts of 4HT. After 48 hours, total cellular lysates were prepared and analyzed by Western blot for MyHC, myogenin and ERK1/2 protein expression. Relative amounts of proteins were quantified by scanning densitometry. MyHC and myogenin protein amounts were normalized to the amount of ERK2 protein. SEM were less than 5% for each measurement.

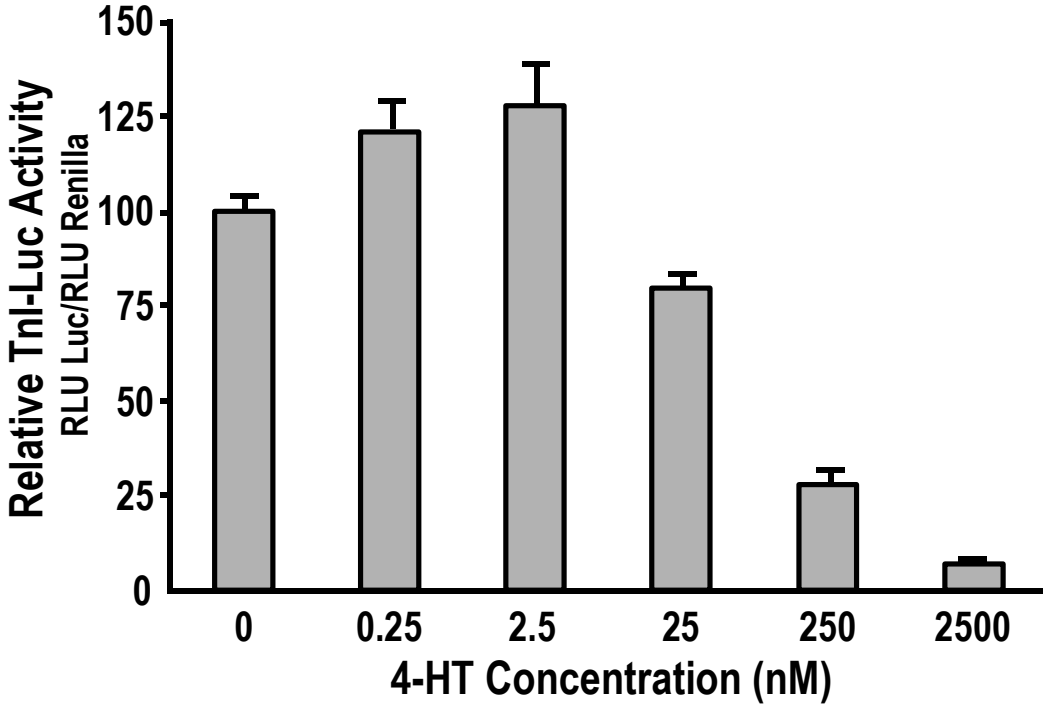

Figure 3.5. *Raf activation alters two distinct phases of myogenesis.* 23A2RafER^{DD} myoblasts transiently transfected with troponin I luciferase (TnI-Luc) and pRL-TK were cultured in differentiation medium supplemented with increasing concentrations of 4HT. TnI-Luc was normalized to *Renilla* luciferase activity. Low-level Raf activity causes a slight increase in muscle reporter gene activity while high-level Raf activity inhibits myogenesis. Data represents the mean and SEM of at least three independent experiments.

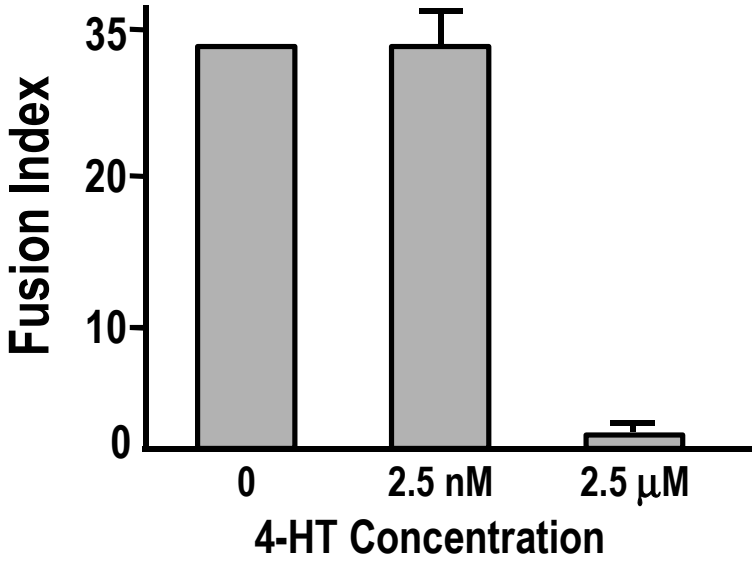

Figure 3.6. *23A2RafER^{DD} conditioned media inhibits myocyte formation.* 23A2RafER^{DD} myoblasts were cultured for 48 hours in differentiation medium supplemented with DMSO, 2.5 nM or 2.5 μM 4HT. Medium was collected and horse serum added to a final concentration of 2%. Conditioned medium was added to confluent cultures of 23A2 myoblasts. Cells were fixed after 48 hours and immunostained for MyHC expression. Fusion Index was calculated as total nuclei contained within MyHC immunopositive myocytes divided by the total number of cells and multiplied by 100. Data represents the mean and SEM of at least three independent experiments.

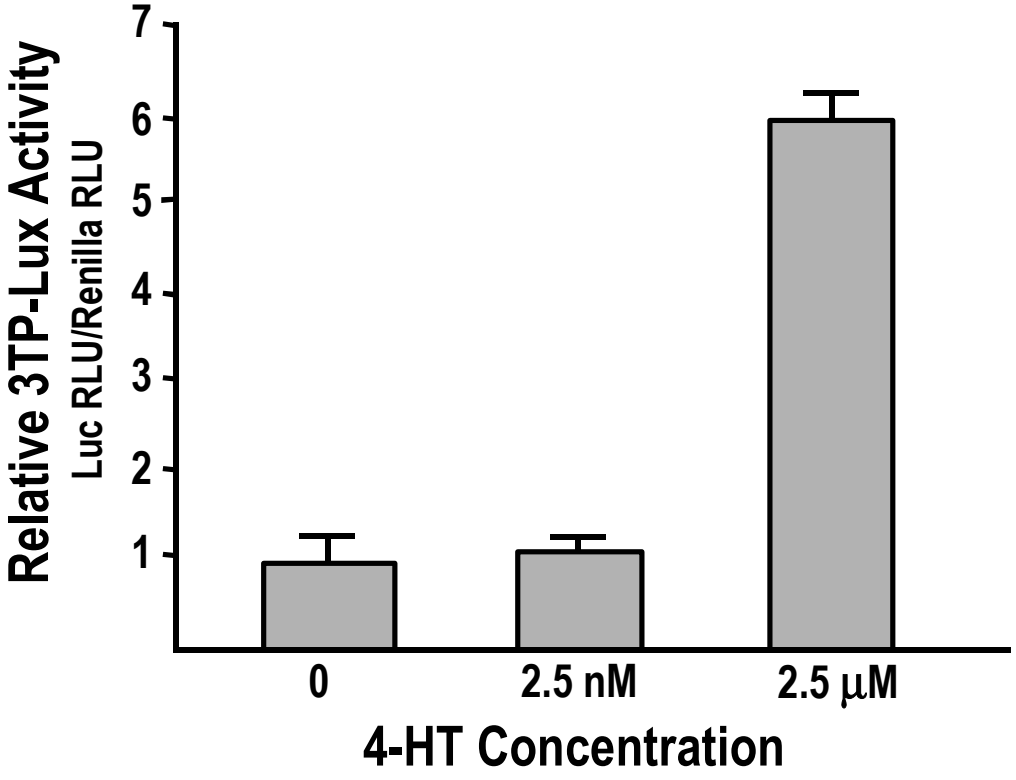

Figure 3.7. *High-level Raf activity increases Smad-directed transcription.* 23A2RafER^{DD} myoblasts were transiently transfected with pRL-TK and 3TP-Lux, a TGF-β responsive promoter reporter gene. Cells were treated for 48 hours in differentiation medium supplemented with 2.5 nM or 2.5 μM 4HT prior to lysis and luciferase measurement. Reporter activity was normalized to *Renilla* luciferase. Data represents the mean and SEM of at least three independent experiments

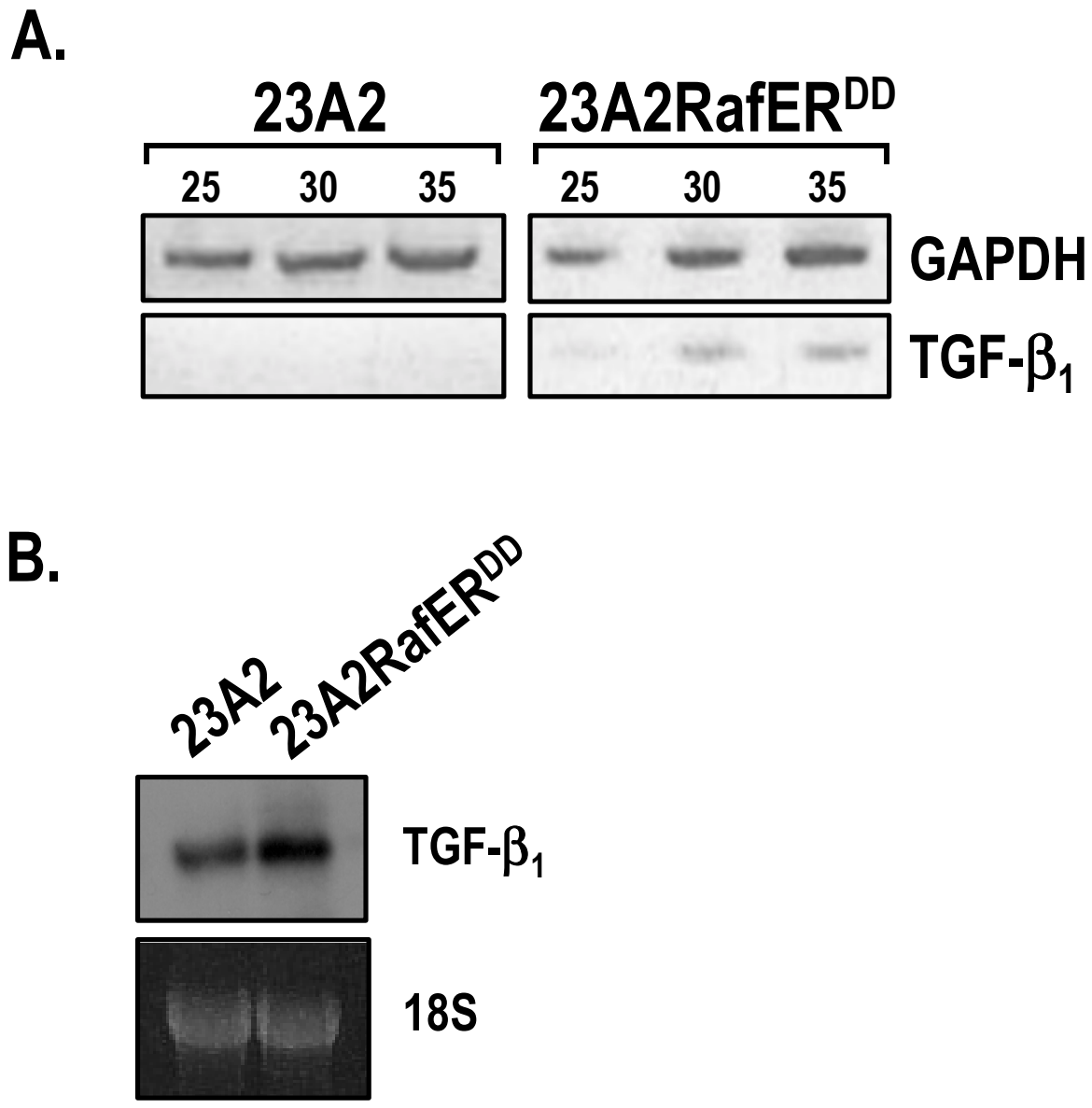

Figure 3.8. *High-level Raf activity up-regulates TGF-b$_1$ gene expression.* 23A2 and 23A2RafER^{DD} myoblasts were differentiated for 48 hours in the presence of 2.5 μM 4HT. Total RNA was isolated and reverse transcribed. PCR amplification was performed using GAPDH and TGF-β_1 gene-specific primers. Five microliters of the amplification reaction (50 μl total) were removed after 25, 30 and 35 cycles (***A***). Total RNA was analyzed by Northern blot using a ^{32}P-TGF-β_1 cDNA probe. Autoradiography demonstrates a specific increase in TGF-β_1 gene expression in 23A2RafER^{DD} myoblasts (***B***).

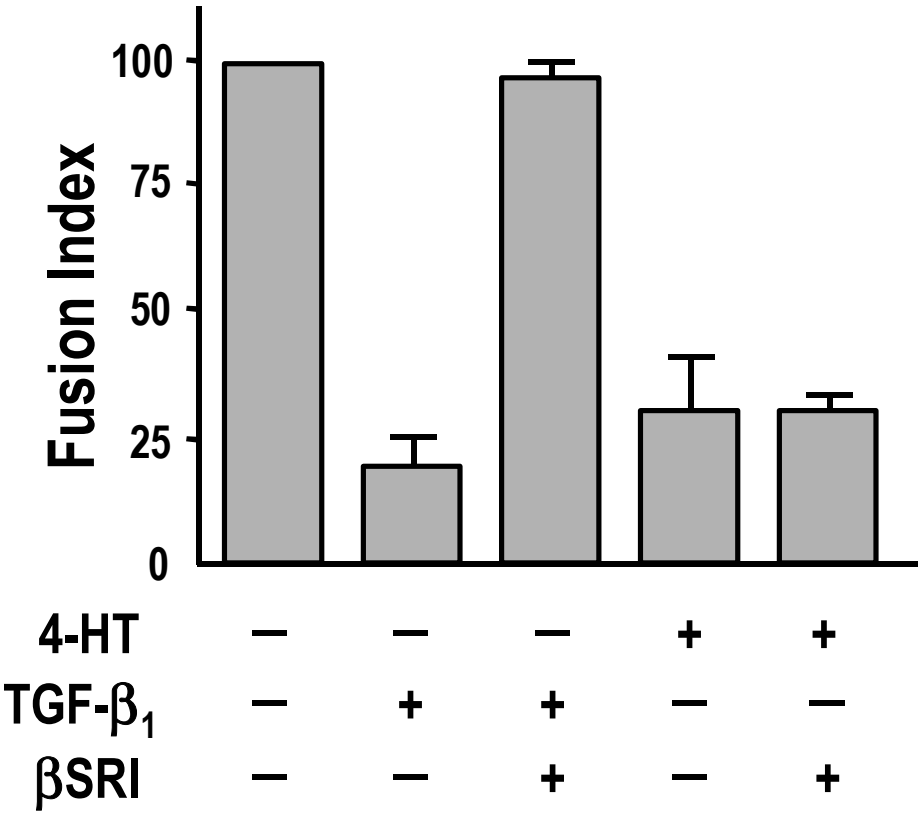

Figure 3.9. *Loss of TGF-β_1 function does not restore myogenesis to 23A2RafER^{DD} differentiation-defective myoblasts.* Confluent cultures of 23A2RafER^{DD} myoblasts were treated with differentiation medium supplemented with DMSO, 2 ng/ml TGF-β_1 or 500 nM 4HT and 2 μg/ml βSRI, an inhibitor of TGF-β function, for 48 hours. Cells were fixed and immunostained for MyHC protein expression. The relative level of myocyte fusion in cells treated with DMSO was set to 100%. Data represents the mean and SEM of at least three independent experiments.

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Refereed Publications

Thomson, S.R. and S.E. Johnson. Isolation and characterization of chicken TaxREB107, a putative DNA binding protein abundantly expressed in muscle. 2001. *Gene* 278: 81-88.

Wang, X., **S.R. Thomson**, J.L. Page, A.D. Ealy, S.E. Johnson. Transforming growth factor beta-1 ($TGF\beta_1$) is upregulated by activated Raf in skeletal myoblasts but does not contribute to the differentiation-defective phenotype. *Submitted to Journal of Biological Chemistry.*

Thomson, S.R., X. Wang, J.L. Page, C.M. Dorman, and S.E. Johnson. A shift in AP-1 components contributes to inhibition of myogenesis through interference with MEF2 function. *Submitted to Journal of Experimental Cell Research.*

Professional Affiliations

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American Physiological Society, 2001-Present