

The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription

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Summary

The expression of Myod is sufficient to convert a fibroblast to a skeletal muscle cell, and, as such, is a model system in developmental biology for studying how a single initiating event can orchestrate a highly complex and predictable response. Recent findings indicate that Myod functions in an instructive chromatin context and directly regulates genes that are expressed throughout the myogenic

program, achieving promoter-specific regulation of its own binding and activity through a feed-forward mechanism. These studies are beginning to merge our understanding of how lineage-specific information is encoded in chromatin with how master regulatory factors drive programs of cell differentiation.

Introduction

Developmental biologists have long recognized the role of cell lineage in establishing the competence of a cell for terminal differentiation. The association of specific chromatin modifications with individual cell lineages – for example, the association of DNase hypersensitivity and histone modifications with the hemoglobin gene in red blood cell nuclei but not in nuclei from other cells (Litt et al., 2001; Weintraub and Groudine, 1976) – suggested that developmentally established chromatin modifications can determine the genes that are available to be expressed in an individual cell-type. A distinction was made between ‘housekeeping’ genes – genes that are constitutively expressed in all cells – and ‘luxury’ genes, such as the hemoglobin locus, the expression of which is restricted in certain cell-types or in certain circumstances (Weintraub, 1972). A crucial question in developmental biology since then has been whether such luxury genes are specifically marked for activation by their inherited chromatin structure, or whether transcription factors that are unique to a differentiated cell type can alone induce luxury gene expression and impose upon cells a chromatin structure, regardless of their lineage-established chromatin components.

To address this question, it was necessary to identify the factors that regulate luxury gene expression in a specific cell-type and then to determine whether they had similar activity in different lineages. It was reasonable to believe such factors could be identified because several studies had already indicated that complex programs of cell differentiation might be regulated by the expression of a very small number of genes, or possibly a single gene, a so-called ‘master switch’ (Holtzer et al., 1975a; Holtzer et al., 1975b; Weintraub et al., 1973). It was in this context that a screen for genes that regulate skeletal myogenesis led to the identification of Myod1 (Lassar et al., 1986), a basic-helix-loop-helix (bHLH) transcription factor that can induce skeletal muscle differentiation in cells from many different lineages.

The identification of Myod as a master regulatory gene of skeletal muscle differentiation provided the opportunity to assess the roles of tissue-specific transcription factors and chromatin-associated proteins in regulating cell differentiation. It also provided the opportunity to address another important question: how does a single transcription factor execute an entire program of cell differentiation? One reason to be interested in the answer to this question is that it is a specific example of the broader question: how does a single event result in a predictable, complex response? It is likely that understanding the orchestration of skeletal muscle gene expression by Myod will provide insights into the regulation of other complex biological events.

This review describes generally how Myod regulates the program of skeletal muscle differentiation: the simple story of a transcription factor activating its target genes [reviews covering other aspects of skeletal muscle development and Myod function, such as cell cycle regulation, have been published previously (e.g. Buckingham et al., 2003; Kitzmann and Fernandez, 2001; McKinsey et al., 2001; Pownall et al., 2002; Puri and Sartorelli, 2000)]. It also describes how a single factor integrates information from co-regulators and from chromatin-associated proteins to achieve promoter-specific binding and promoter-specific transcriptional activation of target genes to temporally pattern gene expression throughout a program of cell specification and differentiation. Based on our current understanding of Myod, it is reasonable to suggest that the regulated activity of a single factor that broadly interacts with many cellular components might be a common mechanism for orchestrating a complex response to an initiating event.

The cloning of Myod, a master switch for skeletal muscle

In 1979, Taylor and Jones demonstrated that treating the mouse fibroblast cell line 10T1/2 with the demethylating agent 5-azacytidine generated clones with a skeletal muscle phenotype

(Taylor and Jones, 1979). This finding indicated that DNA demethylation was sufficient to induce skeletal muscle gene expression in these cells. When genomic DNA was isolated from these muscle clones and stably transfected into untreated 10T1/2 cells, myogenic colonies were generated at a frequency that was consistent with the presence of a single locus that could convert the fibroblasts into skeletal muscle cells (Lassar et al., 1986). The same cell system was then used to clone the cDNA for the myogenic determination gene *Myod1* (Davis et al., 1987), hereafter referred to as *Myod*. When expressed in primary fibroblasts or in a wide variety of other cell types, such as pigment, nerve, fat and liver, *Myod* can convert these cells to skeletal muscle (Weintraub et al., 1989). These findings provided the first direct evidence that a single gene can initiate a complex program of differentiation, acting as a master switch.

It is likely that the azacytidine-mediated demethylation of the *Myod* gene results in the conversion of 10T1/2 cells to skeletal muscle, because the *Myod* gene is heavily methylated and not expressed in 10T1/2 cells but becomes relatively demethylated and is expressed following treatment with 5-azacytidine (Jones et al., 1990). While the demethylation of the *Myod* locus is sufficient to activate its expression in 10T1/2 cells, the *Myod* gene is not methylated in primary fibroblasts and is not expressed in these cells. Heterokaryon formation between primary fibroblasts, which have an unmethylated *Myod* gene, and 10T1/2 cells, which contain the trans-acting factors necessary for the expression of a transfected unmethylated *Myod* gene, did not result in the expression of the unmethylated fibroblast *Myod* gene (Thayer and Weintraub, 1990), indicating that expression of the *Myod* gene is specifically suppressed in primary fibroblasts. It was subsequently shown that the homeobox factor *Msx1* recruits the linker histone H1B to the *Myod* enhancer element to repress its transcription (Lee et al., 2004; Woloshin et al., 1995). Therefore, in at least some primary cell types, *Myod* transcription is actively suppressed by a combination of *Msx1* and linker histones. Interestingly, this suppression is lost in the generation of many fibroblast cell lines, and clones that emerge through crisis have a methylated *Myod* locus that prevents its expression (Jones et al., 1990). This suggests that the unregulated cell divisions that lead to crisis might release the suppression of differentiation genes, like *Myod*, perhaps as a means of limiting growth. Consequently, only clones with a methylated *Myod* locus grow through crisis. It is interesting to speculate that releasing the suppression of *Myod*, and perhaps of other differentiation-related genes, might contribute to the epithelial-to-mesenchymal transition that is associated with the progression of some cancers (Guarino, 1995).

Myogenic bHLH transcription factors and skeletal muscle development

The ability of *Myod* to convert fibroblasts and other cell types into skeletal muscle strongly indicated that it might have a central role in myogenesis, and subsequent studies have sought to determine its biological role in development. The *Myod* protein contains a bHLH motif that is common to a large family of transcription factors (Ledent et al., 2002; Ledent and Vervoort, 2001) (Fig. 1). In addition to *Myod*, the highly related bHLH proteins *Myf5*, *Mrf4* and *Myogenin* (*Myog*) are also expressed in skeletal muscle, and each has a crucial role

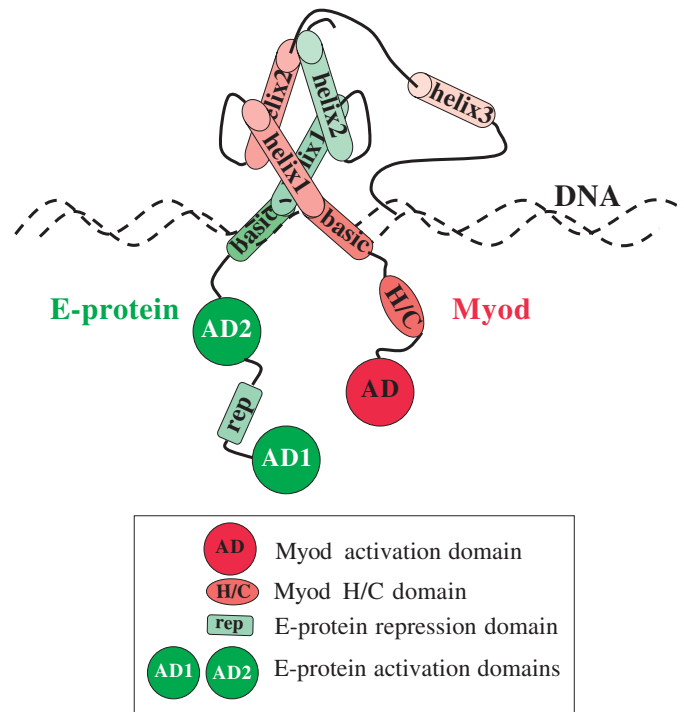


Fig. 1. The functional domains of Myod. Myod (red) forms a heterodimer with an E-protein (green) through the helix-loop-helix domains (helix1 and helix2). The adjacent basic regions (also in an alpha helical conformation) contact the DNA. In Myod, the basic region also contains the 'myogenic code'. This consists of three residues that are conserved in all of the myogenic bHLH proteins (*Myod*, *Myf5*, *Myog* and *Mrf4*), which do not directly affect DNA binding but are necessary to activate the transcription of specific muscle genes by either interacting with co-factors or inducing conformational change, or both. Myod has a single transcriptional activation domain (AD), and a histidine- and cysteine-rich (H/C) region that contains a tryptophan residue that is needed for Myod to interact with the Pbx/Meis complex. The helix 3 region is also required for Myod to cooperatively bind to the Pbx/Meis complex at the *Myogenin* (*Myog*) promoter. The E-protein has two independent activation domains (AD1 and AD2) and a domain that can repress the function of either activation domain (rep).

in muscle cell specification and differentiation (Buckingham et al., 2003; Molkenstein and Olson, 1996; Perry et al., 2001; Pownall et al., 2002; Puri and Sartorelli, 2000), as described below. Understanding the mechanisms by which the myogenic bHLH protein family regulates myogenesis is likely to provide insight into the differentiation of many different cell types, because differentiation in many different lineages is regulated by specific subfamilies of bHLH proteins. For example, analogous to the *Myod* sub-family of bHLH proteins, the *Neurod* sub-family of bHLH proteins is largely restricted to neural and neuroendocrine cells, and regulates neuronal specification and differentiation (Bertrand et al., 2002). The E-protein sub-family of bHLH proteins (*Tcf3*, *Tcf4* and *Tcf12*) has a crucial role in lymphocyte differentiation (Engel and Murre, 2001; Greenbaum and Zhuang, 2002), and its family members also function as heterodimer partners for many of the tissue-restricted bHLH proteins, such as *Myod* and *Neurod* proteins. [For a general review of HLH transcription factors, see Massari and Murre (Massari and Murre, 2000)].

Genetic experiments have shown that either *Myod* or *Myf5* can specify the skeletal muscle lineage in mice: mouse muscle develops relatively normally without either *Myod* or *Myf5*; but disrupting both genes results in the absence of skeletal muscle cells, indicating that these genes are necessary to establish a viable muscle lineage (Rudnicki et al., 1993). *Myog* appears to function 'downstream' of *Myf5* and *Myod* to activate muscle gene expression, and appears to be crucial for the differentiation of muscle cells in vivo. The disruption of *Myog* in mice prevents muscle differentiation in vivo, despite the continued expression of *Myod*, whereas cells cultured from the muscle primordium of *Myog* null mice form skeletal muscle as efficiently as cells derived from wild-type mice (Hasty et al., 1993; Nabeshima et al., 1993). This indicates that the functions of *Myod* in terminal differentiation are suppressed by in vivo signaling events. By contrast, *Myog* is 'blind' to these suppressive signals and executes the differentiation program in vivo. Recent studies indicate that *Mrf4* has roles in both muscle specification and differentiation (Kassar-Duchossoy et al., 2004). (See Fig. 2 for more on the epistatic relationships among the myogenic bHLH genes.)

During normal development, the expression of the myogenic sub-family of bHLH proteins is almost entirely restricted to the skeletal muscle lineage, although *Myf5* is also expressed transiently in some cells in the developing nervous system (Tajbakhsh et al., 1994). Cells in the pre-somitic mesoderm express both *Myf5* and *Mdfi* (Kraut et al., 1998), an inhibitor of *Myf5* also known as I-mfa. It remains unknown whether *Myf5* protein regulates any gene expression in the presomitic mesoderm or whether the inhibitory *Mdfi* effectively blocks its activity. The cells of the presomitic mesoderm that express both genes will ultimately generate the myotome, which is the region of the dermamyotome in the somite that gives rise to skeletal muscle [both epaxial (dorsal) and hypaxial (ventral) muscles]. Cells that express *Myf5* and *Mdfi* will also give rise to sclerotome, the vertebral-cartilage forming part of the somite, and to other somatically derived tissues. In the presomitic mesoderm, therefore, the low expression of *Myf5* is not sufficient to commit the cells to an exclusively skeletal muscle fate. In the dorsal lip of the dermamyotome, however, expression of the inhibitory *Mdfi* decreases and the expression of *Myf5* protein increases (Kraut et al., 1998), co-incident with the specification of the skeletal muscle lineage.

Early expression of *Myf5* is prominent in the epaxial myotome, where it drives the differentiation of the back, intercostal and abdominal wall muscles. By contrast, the early expression of *Myod* is most prominent in the hypaxial myotome, where it drives the differentiation of the limb, tongue and diaphragm muscles, and the muscles of branchial arch-derived tissue (Kablar et al., 1998). Although *Myf5* is not necessary for the expression of *Myod*, the combined deletion of *Myf5* and *Pax3* in mice results in the absence of *Myod* expression (Tajbakhsh et al., 1997), suggesting that these two factors are required to initiate *Myod* expression; however, as discussed below, the elements regulating *Myf5* and *Myod* transcription are complex and remain poorly understood.

Myod and Myf5: nodal points in skeletal muscle specification

It has been well documented that signaling from the surrounding tissues regulates the expression of the myogenic

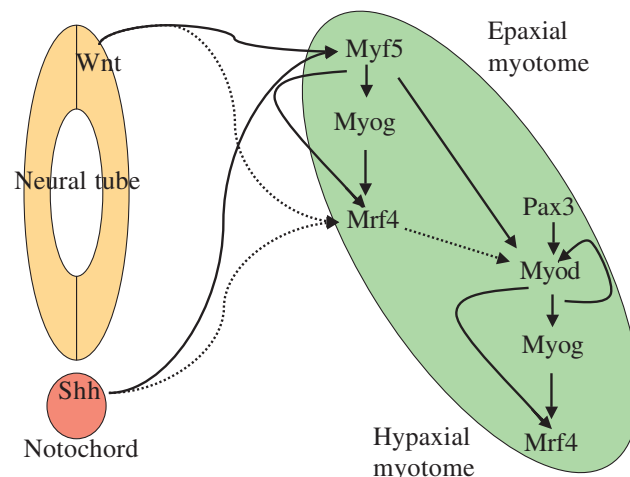


Fig. 2. Epistatic relations among the myogenic bHLH factors. Shh and Wnt signaling from the notochord and dorsal neural tube, respectively, have been shown to regulate the expression of *Myf5* in the epaxial dermamyotome of the somite (green). *Pax3* and *Myf5* independently regulate *Myod* expression. The factors regulating the early expression of *Mrf4* are not known; however, it is likely that the same factors necessary for *Myf5* expression regulate the transient expression of *Mrf4* in the somite (shown as dashed lines) because these genes are physically very close together and share regulatory elements. *Myod* positively auto-regulates its own expression and activates the expression of *Myog*, and both *Myod* and *Myog* are expressed during skeletal muscle differentiation. In addition to its early and transient expression in the somite, *Mrf4* is also expressed in the terminally differentiated muscle cells, and it is likely that *Myod* and *Myog* regulate this late expression of *Mrf4*. A transgene that drives *Mrf4* expression from the *Myog* promoter can partly compensate for the loss of *Myog* (Zhu and Miller, 1997), demonstrating a partly redundant role of *Mrf4* and *Myog* in terminal differentiation.

bHLH genes in the somite: sonic hedgehog (Shh) from the notochord and floor-plate, Wnt signaling from the dorsal neural tube (see Fig. 2), and *Bmp4* signaling from the adjacent lateral plate mesoderm combine to initiate and restrict myogenesis to the muscle-forming region of the dermamyotome (Cossu and Borello, 1999). It is less clear how these signaling events are integrated by the regulatory elements of *Myf5* or *Myod*. Multiple enhancers are spread over hundreds of kilobases in the *Myf5* locus, and each regulates *Myf5* expression at particular developmental times and locations (Buchberger et al., 2003; Hadchouel et al., 2003). An enhancer necessary for epaxial expression has been shown to respond to Gli proteins and might account for the role of Shh signaling in regulating *Myf5* expression (Gustafsson et al., 2002; Teboul et al., 2003). Two enhancer elements have been identified for *Myod*: one is necessary for early myotomal expression, and the other functions slightly later in the myotome and also during the activation of adult muscle satellite cells in muscle regeneration (Asakura et al., 1995; Goldhamer et al., 1995); however, the factors regulating these functions remain largely unknown.

It appears, therefore, that complex signaling events from surrounding embryonic tissues are integrated by complex regulatory elements at the *Myf5* and *Myod* loci to accomplish a simple binary decision: whether or not to express *Myod* or

Myf5. As noted above, these transcription factors are necessary and sufficient for skeletal muscle formation, and the instruction to express either of them effectively specifies the skeletal muscle lineage. In addition, auto-regulation and cross-regulation exists among the myogenic bHLH proteins (Thayer et al., 1989), such that a transient embryonically instructed induction results in stable expression of these factors. The convergence of multiple embryonic signals on this simple binary decision has been referred to as a 'nodal point' in muscle cell specification (Weintraub et al., 1991a). The identification of other bHLH proteins that regulate complex programs of differentiation, such as the Neurod or the E-protein families, suggests that the specification of other cell lineages might rely on similar nodal points to integrate complex instructive signaling into simple binary decisions.

Regulation of Myod binding and activity

Once expressed, how does Myod regulate skeletal muscle cell differentiation? In one sense, the answer seems fairly simple: Myod is a transcription factor with binding sites in the regulatory regions of many genes that are expressed in skeletal muscle. Myod forms heterodimers with the nearly ubiquitous E-protein sub-family of bHLH proteins through the interaction of the HLH domains (see Fig. 1) (Lassar et al., 1991; Murre et al., 1989). The basic regions act as sequence-specific DNA-binding domains that recognize a binding site with the simple core consensus sequence of CANNTG, termed an E-box, and show additional preferences for internal and flanking sequences (Blackwell and Weintraub, 1990). Myod has a single amino-terminal acidic-activation domain, as determined by its fusion to the heterologous DNA-binding domain of the Gal4 protein (Weintraub et al., 1991b), whereas E-proteins have a more complex mix of activation and repression domains (Markus et al., 2002) (see Fig. 1). Therefore, the simple model of the transcriptional activity of the myogenic bHLH proteins is that they activate gene transcription by binding to the E-boxes in the regulatory regions of genes that are expressed in skeletal muscle.

There are several problems with this simple model. First, E-boxes occur frequently in the genome, not just in the regulatory regions of genes expressed in skeletal muscle. Second, the many different sub-families of bHLH proteins recognize the same canonical sequences. For example, the Myod, Neurod and E-protein families can all bind to similar sites: yet Myod makes muscle; Neurod makes neurons; and E-proteins activate genes in B and T cells. Therefore, something must limit the potential of these proteins to promiscuously activate genes. Third, skeletal muscle genes are not all expressed simultaneously. Therefore, temporal specificity and promoter specificity must be superimposed on the simple model of a transcription factor and its binding sites.

Intermolecular interactions appear to be necessary for Myod to activate gene transcription. Myod does not activate reporter constructs with a single E-box but robustly activates reporters with paired E-boxes (Weintraub et al., 1990). This is at least partly due to the fact that Myod forms a relatively stable complex with DNA if two E-boxes are present, whereas there is a fast dissociation rate from a single E-box, indicating that inter-protein interactions stabilize binding, possibly through induced conformational changes. Binding sites for other factors, such as Mef2, Sp1, or Pbx and Meis, can functionally

substitute for the second E-box, indicating that cooperative homotypic or heterotypic interactions with adjacent factors are crucial for establishing a stable and functional transcriptional complex (Biesiada et al., 1999; Knoepfler et al., 1999; Sartorelli et al., 1990). Therefore, the presence of certain binding sites paired with an E-box could confer promoter-specific activity to Myod, or, by extension, to Neurod or the E-proteins, depending on the availability of the cooperating transcription factors.

In addition to cooperative binding, co-factor interaction or sequence-specific DNA/protein interactions might alter the conformation of the Myod complex to effectively expose activation regions. Myod and the other myogenic bHLH proteins have a conserved set of amino acids in the basic region that do not significantly alter the sequence specificity of DNA binding, but do alter the transcriptional activity of the bound Myod (Brennan et al., 1991; Davis et al., 1990). Introducing this amino acid motif into the basic region of an E-protein will convert it into a myogenic protein (Davis and Weintraub, 1992). This myogenic 'code' in the basic region might function by interacting with co-factors – and an interaction with Mef2 factors has been demonstrated (Molkentin et al., 1995) – or these residues might alter the conformation of the bound protein in a manner that presents other regions for co-factor interaction (Bengal et al., 1994; Ma et al., 1994), such as has been suggested for the promoter specific activity of NF- κ B (Leung et al., 2004).

Myod and chromatin remodeling

Lineage-centric models of cell specification focus on the role of chromatin in restricting the response of a cell to transcription factors. Central to this model is the ability of chromatin to suppress the transcription of genes that are extraneous to the specific lineage. For example, in the erythrocyte lineage, hemoglobin expression is associated with an open chromatin structure and hypersensitive sites, whereas in non-erythroid cells, the chromatin at this locus adopts a transcriptionally repressive conformation (Weintraub and Groudine, 1976). The fact that a transiently transfected globin gene is expressed at low levels in non-erythroid cells, whereas the endogenous gene is highly repressed, supports a model in which the chromatin context of a specific gene determines how accessible it is to transcription factors that may be expressed in many different cell types (Wold et al., 1979). Implicit in this model is the need for factors to establish the lineage-specific chromatin context. This could occur: (1) in a lineage-dependent manner, by, for example, the sequential and combinatorial use of factors, such as homeobox and segmentation genes, that are laid down at sites in the chromatin throughout a cell lineage; or (2) in a lineage-independent manner, through the action of a single 'pioneer' transcription factor (Cirillo et al., 2002) that can both access genes in a repressive chromatin context and actively remodel the appropriate loci independent of the prior lineage. The ability of Myod to convert cells of many different lineages and differentiation states to skeletal muscle suggests that it has the characteristics of a pioneer transcription factor; however, as elaborated below, both mechanisms are likely to function in myogenesis.

The first studies in this area sought to determine whether Myod could gain access to genes in native chromatin and initiate chromatin remodeling. Nuclease access studies showed

that genes regulated by Myod, such as *Myog*, muscle creatine kinase (*Ckmm*), and the auto-regulated *Myod* gene itself, were in an inaccessible chromatin context prior to the presence of Myod, and that Myod was able to initiate chromatin remodeling at these loci even in the presence of the protein synthesis inhibitor cycloheximide (Gerber et al., 1997). Myod directly binds the histone acetyltransferase (HAT) p300, and p300 recruits another HAT, the p300/CBP-associated factor (PCAF), to form a Myod complex with two distinct HAT activities (Puri et al., 1997a; Puri et al., 1997b; Sartorelli et al., 1997; Sartorelli et al., 1999). Based on in vitro transcription studies, the two HATs have distinct functions: p300 acetylates histones, whereas PCAF acetylates Myod at lysine residues near its DNA-binding domain; both of these activities are necessary for the full transcriptional activity of Myod on chromatin-associated templates (Dilworth et al., 2004). In addition to recruiting HATs, Myod recruits the Swi/Snf chromatin-remodeling complex through an interaction that can be regulated by the p38 MAP kinase (Simone et al., 2004). Inhibition of HAT activity or inhibition of Swi/Snf activity prevents the ability of Myod to initiate transcription and chromatin remodeling at specific loci (de la Serna et al., 2001; Puri et al., 1997a). In these regards, Myod has the characteristics of a pioneer transcription factor: it can access genes in repressive chromatin and initiate chromatin remodeling through the recruitment of HATs and the Swi/Snf complex.

Myod might also have a repressive role at its target genes prior to initiating chromatin remodeling. Based on chromatin immunoprecipitation (ChIP) studies, Myod is associated with some promoters, such as those of the *Myog* and acetylcholine receptor genes, prior to the onset of differentiation and expression of these genes (Liu et al., 2000; Mal and Harter, 2003). In contrast to the differentiating muscle cell, where Myod is associated with HATs, in the myoblast (the replicating muscle precursor cell), Myod is associated with histone deacetylases (HDACs) and might actively suppress gene expression (Fulco et al., 2003; Mal and Harter, 2003; Mal et al., 2001).

These findings strongly suggest that Myod acts to negatively regulate the transcription of some genes in the myoblast and that muscle differentiation is initiated when Myod switches from its association with repressive factors to activating factors. The differentiation of *Myod*-expressing cell lines, such as the C2C12 myoblast cell line (Silberstein et al., 1986), can be induced in culture by removing serum or it can be prevented by adding growth factors, such as Fgf or Tgf β (De Angelis et al., 1998; Li et al., 1992), indicating that mitogen stimulation in vivo might sustain a myoblast state. In addition, Notch signaling represses both *Myod* transcription and Myod protein activity (Kopan et al., 1994; Nofziger et al., 1999), and probably contributes to regulating differentiation in vivo. It is interesting that while we have identified several mechanisms that might delay myoblast differentiation, such as mitogens and Notch signaling, we do not yet have a good understanding of the events that occur in vivo to overcome these inhibitory signals and to induce differentiation at a specific time and place.

A feed-forward circuit as a quantal step

How does a single transcription factor execute an entire

program of cell differentiation? How does a single event result in a predictable and complex response? Microarray expression studies of cultured C2C12 cells have shown that expression levels of many RNAs change during skeletal muscle differentiation (Delgado et al., 2003; Tomczak et al., 2004). To determine how many of these changes are caused by the expression and activity of Myod, we assessed gene expression changes in fibroblasts with an inducible Myod protein and observed a similarly large number of expression changes with ~5% of the genes tiled on the array (Bergstrom et al., 2002). Many of the RNAs that showed increased expression in response to Myod were muscle-specific genes, such as skeletal muscle myosins and actins, but many were genes expressed in numerous different lineages, such as the Mef2 transcription factors. Surprisingly, some RNAs induced by Myod coded for proteins that inhibit Myod activity, such as the Id proteins (Benezra et al., 1990). This could partly be due to a non-autonomous inhibitory-surround mechanism. For example, the Notch ligand Delta is an early target of Myod and its expression is followed by the expression of the Notch regulated gene *Hes1* (Bergstrom et al., 2002), indicating that *Myod*-expressing cells might inhibit muscle differentiation in their neighbors through the Notch signaling pathway.

Not all genes are simultaneously expressed in response to Myod activation (Bergstrom et al., 2002). Some are induced immediately, whereas others are induced over the next two days of differentiation. In addition, some genes are expressed transiently and some are directly decreased. Interestingly, the cluster of early expressed genes contains most of the genes that encode adhesion molecules and extracellular matrix molecules, including proteases; the intermediate clusters contain most of the transcription factors; and the latest clusters contain most of the myofibril and cytoskeletal proteins that are associated with the contractile function of skeletal muscle. Following the expression of Myod, therefore, the first sets of genes activated might affect cell migration and positioning, followed by the activation of a set of transcription factors; only later in the differentiation program are many of the muscle contractile proteins expressed.

How is the temporal pattern of gene expression established following the activation of Myod? It is appealing to consider a simple cascade model because of the large numbers of transcription factors activated in the intermediate clusters of Myod-responsive genes. For example, Myod initiates the transcription of *Mef2c*, and *Mef2c* might activate a muscle structural gene; however, muscle-specific genes have not been shown to be activated by the expression of *Mef2c* or any other factor in the absence of Myod or another myogenic bHLH factor. Also in contradiction of a rigid cascade model of temporal regulation, ChIP studies have shown that Myod binds directly to the regulatory elements of genes expressed late in the differentiation program, just as it binds to the regulatory elements of genes expressed early in the program (Bergstrom et al., 2002). Therefore, Myod directly regulates genes throughout the program of muscle gene expression, and temporal patterning is achieved by a combination of promoter-specific regulation of Myod binding and activity.

Because Myod initiates the myogenic differentiation program and that program temporally regulates the activity of Myod, it follows that Myod programs the regulation of its own activity. It does this, at least in part, through a feed-forward

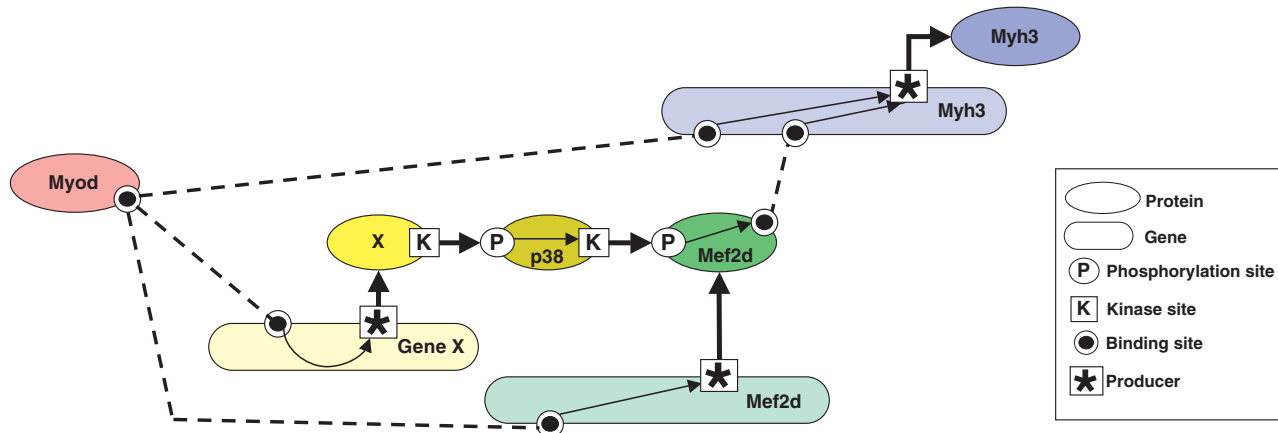


Fig. 3. A Myod-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. Myod regulates the transcription of the Mef2 isoforms, including Mef2d, and activates the p38 kinase pathway, shown here mediated by factor X. Factor X might be the Akt2 kinase, which is transcriptionally regulated by Myod and phosphorylates p38. The phosphorylated p38 becomes an active kinase and phosphorylates Mef2d, permitting it to bind and activate the myosin heavy chain (*Myh3*) gene together with Myod. The *Myh3* gene is not activated by Myod until Mef2d is expressed and p38 is active (Penn et al., 2004). The feed-forward mechanism regulates the activity of Myod at a subset of promoters and imposes a temporal order on Myod-mediated gene expression. This diagram uses the graphical language BioD (Cook et al., 2001).

mechanism (Penn et al., 2004) (Fig. 3). For example, during the first 24 hours after Myod induction in our model system of Myod-mediated myogenesis, Myod initiates expression of the *Mef2d* gene and activates the p38-signaling pathway. Mef2d and p38 then cooperate with Myod to activate a subset of genes initiated between 24 and 48 hours after induction. The precocious activation of p38 and the expression of *Mef2d* permits Myod to activate these normally late genes within hours of induction, demonstrating that the timing of Myod-mediated activation of late genes is imposed by the availability of factors that are activated by Myod at an earlier time-point. In this manner, Myod is active through the entire program of muscle gene expression, binding directly to the regulatory elements of genes expressed both early and late in the program; temporal regulation is achieved by superimposing requirements for additional Myod-regulated factors at subsets of promoters.

One important feature of the feed-forward mechanism is that the entire program is directly regulated by a single factor. The master regulatory protein Myod directly activates genes throughout the program and, in this regard, myogenesis can be considered to be a single-step function. From this perspective, the evolutionary origin of skeletal muscle might have occurred as a single event rather than as a series of individually selected, cascade-type steps that gradually led to the skeletal muscle phenotype (Fig. 4). In this 'quantal step' model, the nearly ubiquitous occurrence of binding sites for bHLH proteins provides the opportunity for these factors to bind throughout the genome. The addition of a new interaction domain to a bHLH factor might broadly alter genome-wide transcription and generate a new selectable phenotype. In this context, the fact that Myod alters the expression of many genes might reflect a basic strategy for generating new cell types, rather than a requirement for the optimal muscle phenotype. In this quantal-step model, one might speculate that the set of genes activated by the original Myod might have lacked the current

temporal patterning, and that the promoter-specific, feed-forward regulation of Myod activity was superimposed during evolution to refine the skeletal muscle phenotype.

This quantal-step model of the evolution of skeletal myogenesis is highly speculative and other models are evident; for example, Myod might have invaded a pre-existing cascade of gene expression and modified its outcome. As we learn more about the skeletal muscle differentiation program, it will be interesting to consider how new cell types and their regulatory circuits evolve. For example, Pha4 regulates pharyngeal development in *C. elegans*, and, like Myod, establishes a temporal pattern of gene expression by binding to and activating the promoters of genes that are expressed throughout the developmental program in a regulated manner (Ao et al., 2004; Gaudet and Mango, 2002; Gaudet et al., 2004). Although it would need to be the subject for a different review, a comparison of vertebrate and invertebrate skeletal myogenesis, and of skeletal and cardiac gene regulatory circuits, might help to inform our understanding of the mechanisms that generate new cell types.

Instructive chromatin and muscle lineage specification

How are Myod and Myf5 capable of accessing the appropriate muscle-specific promoters and specifying the skeletal muscle lineage? As noted above, these factors can recruit histone-modifying and chromatin-remodeling complexes to muscle promoters and reset the cellular chromatin structure and transcriptional program. Rather than acting independently of the pre-existing chromatin, however, recent studies indicate that chromatin-associated complexes instruct these factors about where to bind. As such, chromatin context establishes an instructive environment for the activity of these master regulatory transcription factors.

Several studies led to the characterization of discrete domains in Myod and Myf5 that are necessary to initiate the

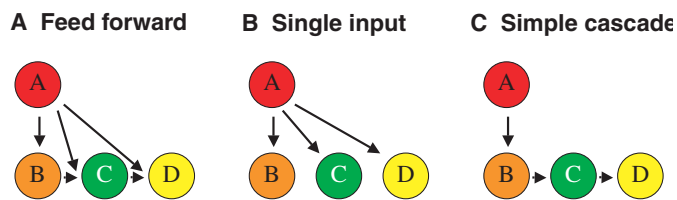


Fig. 4. Evolving a feed-forward regulatory network from a single input or a simple cascade regulatory network. (A) In the feed-forward network, factor A directly regulates each gene: sequential activation is achieved by requiring both A and B to express gene C; and both A and C to express gene D. (B) In a single-input network, factor A directly regulates the three targets B, C and D and does not have temporal patterning. (C) The simple cascade accomplishes sequential gene activation with only gene B directly activated by A. It is easy to see how generating a new single-input network might generate a selectable phenotype that could evolve feed-forward regulation. Evolving a cascade motif would require a selective advantage for each stage, but once it had evolved it could be invaded by factors such as Myod to convert it into a feed-forward network.

myogenic program. When knocked-in to the *Myf5* locus, and in the absence of Myod, *Myog* did not efficiently establish the skeletal muscle lineage in mouse embryos (Wang and Jaenisch, 1997), indicating that Myod and Myf5 have different intrinsic functions to Myog, rather than simply different temporal expression patterns. Subsequently, it was demonstrated that Myod and Myf5 were more efficient than Myog at initiating the expression of a set of endogenous target genes (Bergstrom and Tapscott, 2001). The ability to efficiently initiate endogenous muscle gene expression mapped to two domains conserved in Myod and Myf5, a region rich in histidines and cysteines (H/C domain), which lies immediately N-terminal to the basic region, and a potential amphipathic alpha-helix in the C-terminal region (Helix 3 see Fig. 1). The mutation of the H/C and Helix 3 domains in Myod prevents the initiation of chromatin remodeling at specific target promoters, indicating that they are required prior to the recruitment of an active Swi/Snf complex. The H/C and Helix 3 domains are also conserved in Mrf4, consistent with the recent demonstration that it can also specify the skeletal muscle lineage during embryogenesis (Kassar-Duchossoy et al., 2004).

The conservation of these domains in factors that initiate the skeletal muscle lineage and their necessity for efficiently initiating myogenesis indicate that they have a fundamental role in the molecular mechanism of specifying a cell lineage. Expression array analysis that compared the activity of wild-type Myod with H/C and Helix 3 mutants showed that the expression of ~10% of Myod-regulated genes depends on these two domains, including the *Myog* promoter (Berkes et al., 2004); this study also determined that an interaction takes place between these Myod domains and the homeobox complex of Pbx and Meis at the *Myog* promoter. Interestingly, an earlier study had shown that Myod binds cooperatively with Pbx/Meis through a conserved tryptophan in its H/C region when an E-box is adjacent to a Pbx/Meis site (Knoepfler et al., 1999). In the *Myog* promoter, the Pbx/Meis site is associated with two over-lapping, non-canonical E-boxes (CAACAG and CAGCTT), and both the H/C and Helix 3 regions are required for Myod to form a complex on this site with Pbx/Meis. These

two domains are apparently necessary to alter the conformation of the bound Pbx/Meis, because the H/C and Helix 3 Myod mutants bind as well as wild-type Myod does to the non-canonical E-boxes in the absence of the Pbx/Meis complex. Pbx/Meis binding, therefore, somehow obscures the non-canonical E-boxes unless the H/C and Helix 3 domains are present on Myod (Berkes et al., 2004).

Pbx is bound to the *Myog* promoter in both muscle and non-muscle cells, and it is possible that the interaction between Myod and the Pbx complex is necessary for Myod to initially locate the *Myog* gene within condensed chromatin prior to differentiation. The *Myog* promoter contains a conserved consensus E-box, 100 bp promoter proximal to the Pbx site, which is necessary for full transcription (Berkes et al., 2004; Cheng et al., 1993). However, despite the presence of this intact consensus E-box, ChIP studies show that the Pbx-interacting domains of Myod are necessary to stably recruit Myod to the *Myog* promoter (Berkes et al., 2004). It appears, therefore, that Myod needs to interact with Pbx/Meis and the adjacent non-canonical E-boxes before it can form a stable binding complex at the consensus E-box.

Indeed, Myod targets chromatin-remodeling complexes to the *Myog* promoter prior to forming a stable DNA-bound complex (de La Serna et al., 2005). A Myod-dependent histone acetylation is the initial event at the *Myog* promoter, followed by Swi/Snf recruitment, and then binding of Myod and Mef2 factors. Therefore, an attractive and consistent model is that the canonical E-box is 'hidden' from Myod and other bHLH factors by chromatin in non-muscle cells and that Myod is initially recruited to this locus through an interaction with Pbx (Fig. 5). As noted above, the HATs p300 and PCAF form a complex with Myod, and tethering Myod to the *Myog* promoter results in local histone acetylation. The acetylated histones could then stabilize the binding of the Swi/Snf complex, which is recruited by Myod in a p38-dependent complex. Remodeling of the locus would expose the canonical E-boxes and binding sites to other factors, such as the Mef2 and Six proteins, leading to the formation of a stable multi-protein regulatory complex. Although this complex might be similar to the enhancesome described at the IFN- β promoter (Agalioti et al., 2000), the formation of the IFN- β enhancesome occurs as a first step on exposed DNA with subsequent chromatin remodeling, whereas the emerging model at the *Myog* locus indicates that transcription factor-directed chromatin remodeling must occur before the cognate binding sites are exposed and a stable complex forms on the promoter.

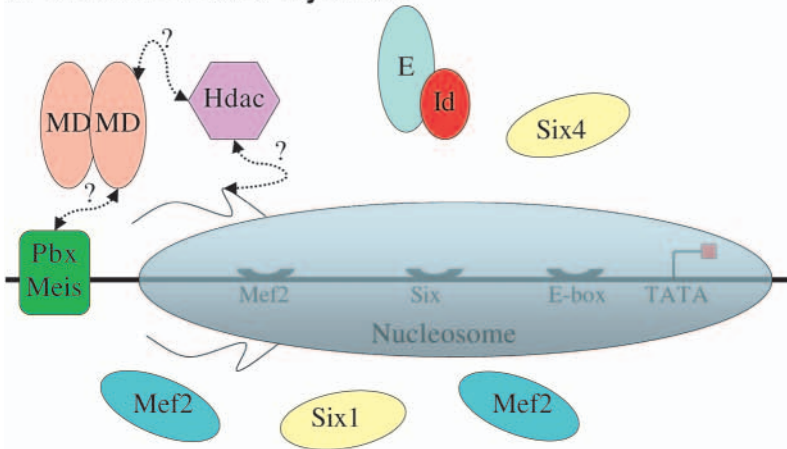
The requirement for an interaction between Myod and the resident Pbx complex to reveal the other binding sites might explain why E-proteins or Neurod do not bind and activate the *Myog* promoter in vivo, despite the fact that they can initiate gene expression at other loci when effecting their own program of differentiation. For example, the E-boxes in the IgH enhancer are nearly identical to the E-boxes in the muscle creatine kinase enhancer, and Myod binds with equally high affinity to both sets of E-boxes in gel shift assays (Kadesch et al., 1986). In addition, Myod can bind and activate the expression of reporter constructs driven by the IgH E-boxes in transient transfection assays. By contrast, despite the ability of Myod to recruit chromatin modifying complexes and initiate chromatin remodeling at many loci, it does not bind to the IgH E-boxes in vivo and does not initiate IgH expression

(Bergstrom et al., 2002). Therefore, chromatin-mediated repression can partly explain the paradox of lineage-specific

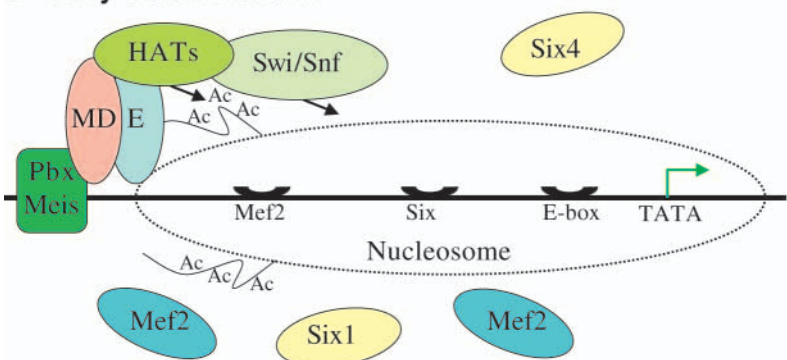
gene activation among a family of factors that bind similar DNA sequences.

Myod and Myog have very similar DNA-binding domains and generally bind the same sequences in gel shift assays. Indeed, global ChIP analysis indicates that Myod and Myog bind to many of the same regulatory sites (Blais et al., 2005), although a subset of sites might be specific to either Myod or Myog. Outside the bHLH domain, Myod and Myog show significant divergence that would permit them to interact with different factors at a promoter. As noted above, the Myod Helix 3 region is required for Myod to initiate binding at some sites by interacting with Pbx. Myog also has an amphipathic alpha helix in its C terminus – the hydrophobic surface is identical in both proteins, whereas the hydrophilic surface diverges significantly. This domain of Myog can function as an activation domain, whereas the Helix 3 of Myod does not have activation domain function (Bergstrom and Tapscott, 2001). These different roles of the Helix 3 region might account, in part, for the different developmental roles of Myod and Myog. The Myod Helix 3 is critically important for the ability of Myod to find genes in chromatin and to initiate chromatin remodeling, but it does not have a specific activation function; the Myog Helix 3, however, brings a new activation domain to

A Undifferentiated myoblast



B Early differentiation



C Late differentiation

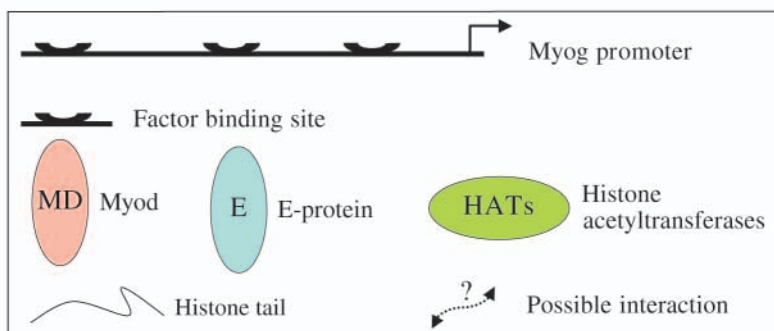
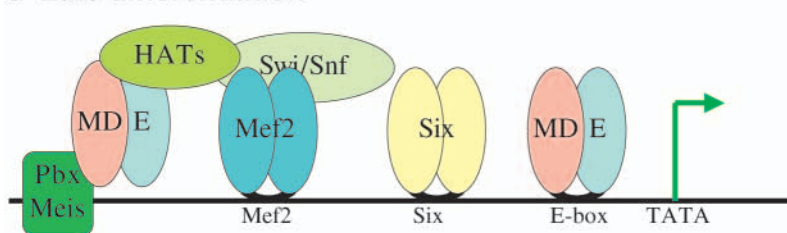


Fig. 5. Myod targets chromatin-remodeling complexes to the Myog promoter. (A) In the undifferentiated myoblast, a nucleosome (gray) is likely to be positioned over the E-box and the binding sites for Mef2 and Six factors, based on the limited access that restriction endonucleases have to this region (Gerber et al., 1997); however, Pbx/Meis is bound even in the presence of the nucleosome (Berkes et al., 2004). Chromatin immunoprecipitation (ChIP) analysis indicates that Myod (MD) recruits an Hdac to the *Myog* promoter in myoblasts (Mal and Harter, 2003), possibly by interacting with the Pbx/Meis complex. Id proteins are expressed in the myoblast and dimerize with E-proteins, preventing the formation of Myod/E-protein heterodimers (Jen et al., 1992). Mef2 isoforms are present but are probably not bound to the *Myog* promoter in the myoblast (de La Serna et al., 2005), and the same is likely to be true for the Six proteins. (B) Early on during differentiation, Id levels decrease, leading to the formation of Myod/E-protein heterodimers that interact with the Pbx/Meis complex at the *Myog* promoter (Berkes et al., 2004; de La Serna et al., 2005). Myod recruits HATs and the Swi/Snf complex (de La Serna et al., 2005; Simone et al., 2004), which acetylate the histones and remodel the nucleosome, respectively. (C) The remodeling of the nucleosome permits Mef2 and Six protein isoforms to access their cognate sites and the stable binding of Myod to its E-box. In this model, therefore, Myod-directed chromatin remodeling must occur before Myod and other factors can access their cognate sites in the promoter. E, E-protein; Ac, acetylation of histone tail; E-box, Myod-binding site; HATs, histone acetyltransferases; Hdac, histone deacetylase.

remodeled genes but keeps Myog 'blind' to genes that have not been remodeled (Bergstrom and Tapscott, 2001; Berkes et al., 2004). An alternative, but not exclusive, role of the Myog Helix 3 region might be to interact with other Pbx-like factors to allow Myog to initiate its own set of genes. Future studies will need to address these possibilities.

Conclusion: wiring the circuitry of a master switch

Myod and the myogenic bHLH proteins are master regulatory genes of skeletal muscle differentiation: they are necessary for skeletal muscle specification, are sufficient to establish the myogenic program, and they directly regulate gene expression throughout the differentiation program. Clearly, however, these master regulatory factors do not act in isolation. There is now evidence that an instructive chromatin environment is developmentally established, as evidenced by the emerging role of the Pbx/Meis complex in marking loci for Myod activation. In addition, the binding and activity of Myod is regulated by other factors to achieve a temporal patterning of gene expression through a feed-forward mechanism. It is likely that other master regulatory factors, such as Neurod in neurogenesis and E-proteins in lymphocyte differentiation, regulate and are regulated through similar mechanisms: a simple DNA-binding site that permits a large sampling of the transcriptional potential of the genome, and super-imposed promoter-specific regulation to achieve a coherent pattern of gene expression.

It is interesting to contrast the role of Myod in muscle cell differentiation with that of Pax6 in eye development, both of which have been termed master regulatory genes (Pichaud and Desplan, 2002; Weintraub et al., 1991a). Myod regulates the differentiation of a single cell type, whereas Pax6 is essential for the development of a complex organ comprising multiple different specialized cell types. Within the context established by Pax6 and by the other homeobox factors of the retinal determination gene network (RDGN) (Silver and Rebay, 2005), transcription factors that regulate cell differentiation in many regions of the body generate the specialized neurons, glia and melanocytes that compose the retina. Relative to our current understanding of the role of Pbx in creating an instructive environment for Myod, it is attractive to think that Pax6 and other factors of the RDGN might establish a chromatin context that modulates the specific activities of the bHLH and other transcription factors to generate the distinct cell types of the eye, similar to the role of Pax and Hox proteins in regulating bHLH activity proposed by Westerman et al. (Westerman et al., 2003).

To extend this speculation, perhaps Pax3 and Pax7 also have instructive roles in Myod-mediated myogenesis. Forced expression of Pax3 in mouse fibroblasts does not activate muscle gene expression, but expressing the PAX3-FKHR fusion protein associated with alveolar rhabdomyosarcomas, a fusion protein that adds FKHR (Forkhead) regulatory domains to the PAX3 DNA-binding domain, activates a large number of skeletal muscle genes, including *Myod*, *Myog*, and muscle structural genes (Khan et al., 1999). Perhaps Pax3 and Pax7 reside at the regulatory regions of subsets of genes expressed in skeletal muscle but do not directly regulate transcription in their native state, similar to the role we are postulating for the Pbx complex at the *Myog* promoter. If this is the case, it might be necessary to remove or replace these factors prior to

differentiation, because *Pax3* and *Pax7* expression ceases at the time of muscle differentiation and forced expression actually inhibits muscle differentiation. If, in the future, we learn that the Pax genes mark regions for a subsequent set of homeobox genes, such as Pbx or Meis, and, in turn, that these instruct Myod or other 'master regulatory factors', then we will have melded the concepts of lineage-established chromatin-encoded potential with master regulatory factor-driven programs of cell differentiation.

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