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Regulating a master regulator

Establishing tissue-specific gene expression in skeletal muscle

Arif Aziz[1], Qi-Cai Liu[1] and F. Jeffrey Dilworth[1,2,*]

[1]Sprott Center for Stem Cell Research; Regenerative Medicine Program; Ottawa Hospital Research Institute; [2]Department of Cellular and Molecular Medicine; University of Ottawa; Ottawa, ON Canada

MyoD is a master regulator of the skeletal muscle gene expression program. ChIP-Seq analysis has recently revealed that MyoD binds to a large number of genomic loci in differentiating myoblasts, yet only activates transcription at a subset of these genes. Here we discuss recent data suggesting that the ability of MyoD to mediate gene expression is regulated through the function of Polycomb and Trithorax Group proteins. Based on studies of the muscle-specific *myog* gene, we propose a model where the transcriptional activators Mef2d and Six4 mediate recruitment of Trithorax Group proteins Ash2L/MLL2 and UTX to MyoD-bound promoters to overcome the Polycomb-mediated repression of muscle genes. Modulation of the interaction between Ash2L/MLL2 and Mef2d by the p38α MAPK signaling pathway in turns provides fine-tuning of the muscle-specific gene expression program. Thus Mef2d, Six4 and p38α MAPK function coordinately as regulators of a master regulator to mediate expression of MyoD target genes.

Key words: chromatin, transcription, muscle, MyoD, p38 MAPK, Mef2, Six4, gene expression

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*Correspondence to: F. Jeffrey Dilworth; Email: jdilworth@ohri.ca

Introduction

MyoD is a master regulator of skeletal myogenesis[1] due to its ability to initiate the myogenic program in myoblasts,[2] fibroblasts[3] and a variety of other cell types.[4] Upon conditions permissive to myogenesis, MyoD heterodimerizes with the more ubiquitously expressed E-proteins[5] to establish a specific and temporally ordered gene expression program[6] giving rise to multinucleated myotubes. This muscle-specific gene expression program is initiated by MyoD and proceeds via a feed-forward mechanism[7] to activate genes which possess the consensus E-box sequence VCASCTG (where V is A, C or G while S is C or G) within their promoter/enhancer regions.[8-11] Reporter assays performed in vivo[12] and in vitro[13,14] demonstrate that MyoD directs high level expression of artificial templates containing multimerized E-boxes. This strong transactivation potential is likely empowered through its ability to interact with multiple transcriptional regulatory factors. Indeed, once associated with the promoter region, MyoD recruits the acetyltransferases p300 (leading to acetylation of histones H3 and H4),[11,15-18] and pCAF (which acetylates MyoD).[14,19] MyoD can also interact with the basal transcriptional machinery,[20,21] the ATP-dependent chromatin remodeling factor SWI/SNF,[17,22,23] the arginine methyltransferase PRMT5,[24] and the transcriptional elongation stimulating factor pTEFb.[25,26] Its ability to interact with a number of ubiquitously expressed transcriptional regulators provides insight into the mechanism through which MyoD activates the muscle-specific gene expression program in multiple cell types. However, recent genome-wide analysis has revealed that the binding of MyoD to specific promoters or enhancers is not sufficient to activate gene expression. Indeed, high-throughput ChIP-Seq analysis performed in differentiating myoblasts indicates that MyoD binds to ~25,000 sites throughout the genome, while only 1,953 genes demonstrate modified expression

during myogenesis.[11] The limited number of differentially expressed genes observed in response to extensive MyoD occupancy at genomic loci suggests that this master regulator of gene expression must itself be regulated to orchestrate the temporally ordered muscle-specific transcriptional program.

While the mechanism by which the transcriptional activity of MyoD is regulated on a genome-wide scale remains poorly defined, recent studies have implicated Polycomb Group (PcG) and Trithorax Group (TrxG) proteins in regulating MyoD activity to ensure proper temporal and spatial expression of muscle genes.[16,17,27,28] For example, YY1 binds to promoters/enhancers of the muscle-specific *MHCIIb* and *CKm* genes to mediate the recruitment of the PcG methyltransferase Ezh2, which establishes the repressive histone H3 lysine 27 trimethyl (H3K27me3) mark across these loci.[27] The dominant nature of the PcG-mediated H3K27me3 mark may play an important role in ensuring that MyoD is unable to activate transcription in the absence of the antagonizing activity of TrxG proteins[29-31], thereby limiting the number of loci expressed in response to MyoD binding. While genome-wide studies of H3K27me3 at different stages of myogenesis have not yet been performed, it is clear that multiple muscle-specific genes are marked for repression by PcG family members in both growing myoblasts[27,28] and fibroblasts.[32] Based on these observations, we tested the hypothesis that TrxG proteins antagonize PcG-mediated repression of muscle loci by removing the repressive H3K27me3 mark and replacing it by a transcriptionally permissive histone H3 lysine 4 trimethyl (H3K4me3) mark.

To understand how TrxG proteins antagonize PcG-mediated repression in muscle, the *myogenin* (*myog)* gene represents an excellent model since a DNA fragment consisting of the -133 to +18 bp region of the *myog* promoter is sufficient to confer muscle-specific expression of a lacZ reporter in transgenic mice.[33,34] Using the *myog* promoter as a model system, we found that the homeobox transcription factor Six4 and the MADS-box transcription factor Mef2d collaborate with MyoD to establish transcriptional competency at this gene.[16,28] Furthermore, we showed that the role of Six4 is to recruit the histone H3K27 demethylase UTX to mediate a localized demethylation of H3K27me3 within the promoter of the gene.[28] Indeed, knockdown of Six4 in differentiating myotubes prevents recruitment of UTX to the *myog* promoter and inhibits the demethylation of H3K27me3 at this locus.[28] Further supporting a role for Six4 in overcoming the repressive effects of PcG proteins is the observation that mutation of the Six4 binding site in the minimal *myog* promoter leads to loss of lacZ expression in transgenic mouse embryos.[35] Taken together, these results provide a mechanism to explain recent findings that Six4 acts synergistically with MyoD to activate the *myog* promoter during myogenesis.[36]

While Six4 mediates removal of the H3K27me3 mark, the role of Mef2d is to permit marking of the *myog* gene with the transcriptionally permissive H3K4me3 modification. Indeed, using a combination of in vitro and in vivo techniques we showed that Mef2d recruits the Ash2L/MLL2 methyltransferase complex to the *myog* promoter thereby allowing the methylation of H3K4 to permit transcriptional activation.[16] Interestingly, we found that the interaction between Ash2L/MLL2 and Mef2d is not constitutive, but instead is the result of a highly regulated cell signaling mechanism that involves direct phosphorylation of Mef2d by the p38α MAP kinase (MAPK).[16] Further supporting a major role for p38α-mediated phosphorylation of Mef2d in the activation of muscle-specific genes is the finding that precocious expression of Mef2d and activated MKK6 kinase (which activates p38 MAPK signaling) in fibroblasts undergoing MyoD-induced skeletal myogenesis leads to early expression of several MyoD target genes.[7] The fact that recruitment of Ash2L/MLL2 to muscle-specific promoters depends on p38α-mediated phosphorylation of Mef2d provides insight into the mechanism by which p38α increases transcriptional activity of Mef2 family members[37] and is consistent with the critical role of this kinase in promoting myogenesis.[38-40] Furthermore, it provides an explanation for the observation that Mef2d-mediated recruitment of RNA Pol II is independent of p38α MAPK signaling although it is necessary for transcriptional elongation at muscle genes.[7] Interestingly, while Ash2L/MLL2 interacts most strongly with phosphorylated Mef2d, this TrxG complex can also interact with phosphorylated Mef2c, but not phosphorylated Mef2a.[16] This variable ability of different Mef2 isotypes to interact with Ash2L/MLL2 suggests some degree of functional specificity between the three members of the Mef2 family of transcriptional activators expressed in muscle. Taken together, these findings have revealed several levels of regulation to ensure proper spatial and temporal patterning of muscle-specific gene expression.

Consistent with our studies demonstrating a cooperation between MyoD, Six4 and Mef2d in the activation of the *myog* gene,[16,28] genome-wide studies by Cao et al.[11] identified enrichment of Mef2 binding sites at MyoD target genes undergoing transactivation, but not at genes whose expression remains unchanged during myogenesis. Similarly, ChIP-microarray studies have identified enrichment for both Mef2 and Six4 binding elements adjacent to muscle regulatory factor bound E-boxes during myogenesis.[10] These findings further support an important role for Mef2 and Six4 as key modulators of MyoD transcriptional activity, allowing this master regulator of myogenesis to overcome PcG-mediated transcriptional repression at specific loci to establish the muscle gene expression program.

To explain the activation of MyoD-dependent transcription during myogenesis, we propose the following model (**Fig. 1**). In a first step the binding of MyoD, Six4 and Mef2d within the transcriptional regulatory region of muscle-specific genes leads to the formation of a poised promoter,[41] which is loaded with the general transcriptional machinery,[20,21] RNA Pol II,[7] the acetyltransferases p300 and pCAF[15-17] and the histone demethylase UTX.[28] The presence of p300 leads to acetylation within the nucleosomes adjacent to MyoD binding sites[15-17] while UTX mediates a localized demethylation of H3K27me3 within the promoter/enhancer region of the gene.[28] Meanwhile, the coding region of the gene remains

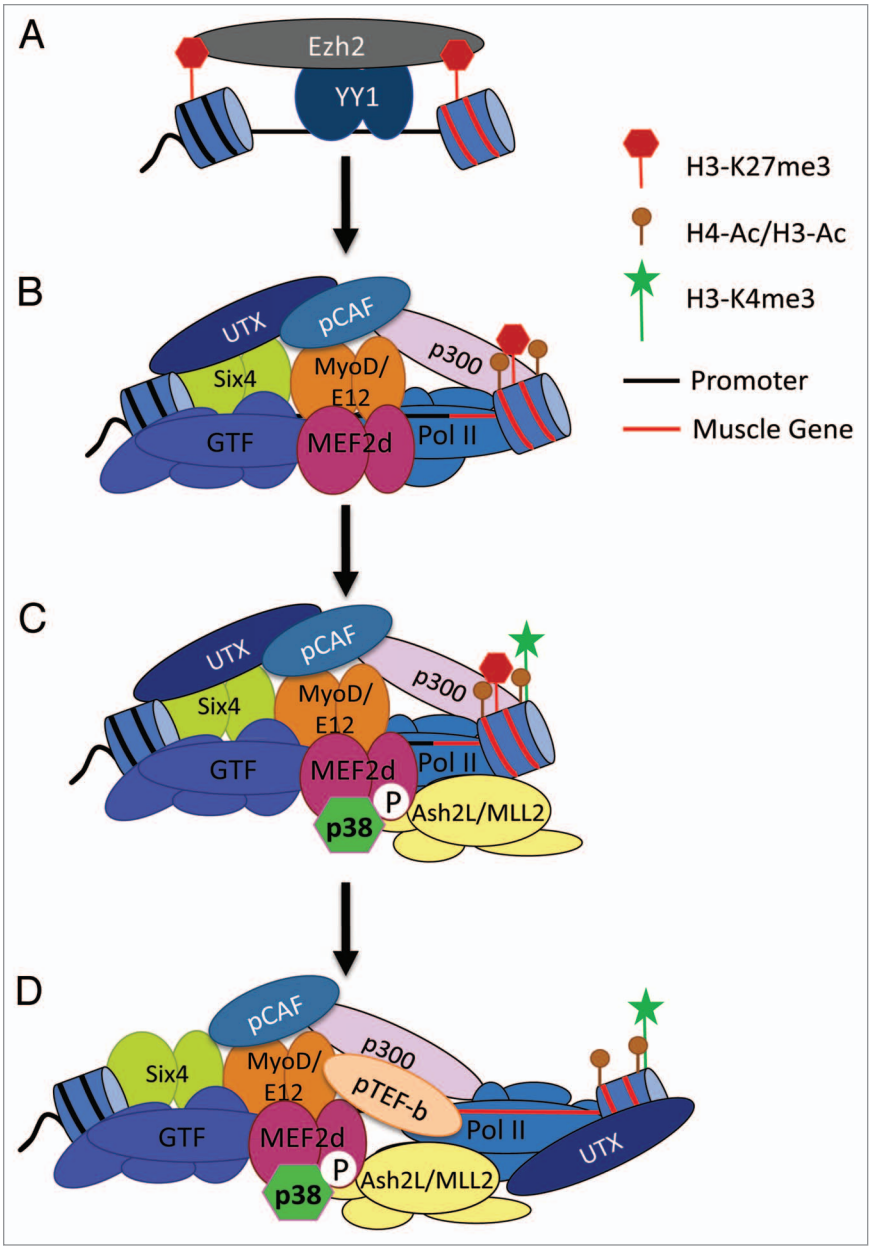

Figure 1. Model for the coordinate activation of PcG-repressed muscle genes. (A) The transcriptional repressor YY1 targets Ezh2 to muscle-specific genes, establishing the repressive H3K27me3 mark across the locus. (B) MyoD binding at the promoter, in conjunction with Mef2d and Six4, establishes a transcriptionally poised promoter characterized by a localized demethylation of H3K27me3 (limited to the promoter) and the presence of acetylated histones. However, transcriptional competency is not achieved due to the presence of repressive H3K27me3 mark within the gene. (C) Phosphorylation of Mef2d by p38 MAPK allows the recruitment of Ash2L/MLL2 complex leading to H3K4me3 within the gene. (D) Phosphorylation of the CTD of RNA Pol II allows the transfer of UTX onto the elongating polymerase to mediate demethylation into the gene, permitting muscle-specific gene expression. See text for further details.

enriched for the repressive H3K27me3 mark, preventing transcription.[28] Upon cell-cell contact, activation of the MAPK signaling pathway (possibly via CDO receptor mediated cascades)[42] results in p38α-directed phosphorylation of the transactivation domain of Mef2d.[7,16] Phosphorylated Mef2d in turn recruits Ash2L/MLL2-containing methyltransferase complexes to the promoter, leading to formation of the transcriptionally permissive H3K4me3 mark at the 5'-end of the gene.[16] The transcriptionally permissive mark then allows engagement of the RNA Pol II and phosphorylation of its C-terminal domain (CTD) at Serine2 via MyoD-dependent recruitment of pTEF-b.[26] Upon phosphorylation of the CTD, UTX associates with the elongating polymerase[43] and removes the repressive H3K27me3 mark across the coding region of the gene.[28] According to this model the ability of MyoD to activate gene expression is regulated by the antagonistic activities of PcG (Ezh2) and TrxG (Ash2L/MLL2 and UTX) through a mechanism mediated by the transcriptional activators Mef2d, Six4 and modulated by p38α MAPK signaling. It is interesting to note that recruitment of the TrxG protein complex SWI/SNF to the *myog* promoter is also modulated by p38α MAPK signaling.[17] The functional relationship between these two TrxG protein complexes (Ash2L/MLL2 and SWI/SNF) at the *myog* promoter remains to be determined.

Our finding that muscle-specific expression of *myog* is directed through a Mef2d-dependent mechanism is consistent with transgenic mouse studies demonstrating that mutation of the Mef2 site within the proximal *myog* promoter inhibits expression in limb buds and a subset of cells in the somite myotome at day 11.5 p.c.[33,34] However, this promoter element is not required for expression of *myog* in somites anterior to somite 10 at this stage of development. Furthermore, by day 12.5 p.c. expression of the transgene is observed in the limb buds.[33,34] There are two possible explanations for this finding. The first possibility is that the same Mef2 element is required for recruitment of the transcriptionally repressive PcG complex onto the muscle-specific promoter at specific stages of development. In that case, the lack of PcG protein recruitment may negate the requirement for TrxG to establish transcriptional competence.[44] However, since expression of *myog* was not observed outside the myogenic lineages, it is more likely that at different developmental stages, alternate transcriptional activators are responsible for recruiting the Ash2L/MLL2-containing methyltransferase complexes to activate the *myog*

promoter in a spatially restricted manner. Indeed, several transcription factors can recruit the Ash2L/MLL2 complex to specific genes. For example, in activated satellite cells Ash2L/MLL2 is recruited to the *Myf5* promoter by the transcriptional regulator Pax7.[45] Since the transgenic mouse studies showing muscle-specific reporter expression in the absence of the conserved Mef2 binding site used an extended *myog* promoter of 1,565 bp, it is currently unclear which additional DNA binding sites are involved in recruiting Ash2L/MLL2-containing methyltransferase complexes to the *myog* gene at alternate spatio-temporal stages. It is important to note that the use of alternate DNA sequences to promote expression of a muscle-specific gene in distinct regions of the embryo is not without precedence. Indeed, extensive mapping of the *Myf5*/*Mrf4* locus during murine development has led to the identification of multiple enhancer elements which act to modulate expression of Myf5 within the muscle cell lineage at specific developmental stages.[46] Future studies looking at the recruitment of the Ash2L/MLL2 complex to *myog* promoter in muscle at different stages of development will be facilitated by advances in chromatin immunoprecipitation (ChIP) protocols which currently require less than 100 cells.[47]

While we are beginning to understand how tissue-specific gene expression is regulated, it remains to be determined how a high level of gene expression is established at particular loci. One consideration is the cell-type specific nuclear reorganization that occurs during differentiation.[48,49] Recent studies in *C. elegans* show that the muscle-specific *Myo-3* promoter localizes to the nuclear envelope in non-muscle cells while shifting to the nuclear lumen where it is transcribed at high levels in cells of the muscle lineage.[50] While muscle-specific expression of the *Myo-3* transgene is observed in the nuclear periphery at early stages of differentiation, transcriptional output from the locus is greatly increased upon transition to transcription factories located in the nuclear lumen.[50] Based on these observations, it is interesting to note that while the -133 to +18 bp region of the *myog* promoter is sufficient for muscle-specific gene expression, high level expression of the transgene is only observed when a region of -1,092 to +18 of the promoter is included in the construct.[33,34] This suggests the existence of a transcriptional enhancer in the region upstream of the promoter, which may facilitate incorporation of the *myog* gene into the transcription factories of the nuclear lumen. The mechanism by which cells undergo extensive nuclear reorganization during myogenesis is not clear. An interesting possibility was suggested by a recent study demonstrating a critical role for the caspase activated nuclease (CAD) in the myogenic process whereby it generates transient DNA strand breaks.[51] It is enticing to propose that the role of CAD in myogenesis could be to facilitate reorganization of chromosomes in the context of the densely packed nucleus.

To conclude, we propose that while MyoD binds to a large number of sites throughout the genome, its ability to regulate transcription is restricted by the additional requirement for Six4 and Mef2d to overcome the repressive effects of PcG-mediated histone methylation. The need for MyoD, Six4 and Mef2d to interact at specific promoters suggests a mechanism by which spatial and temporal regulation of skeletal muscle gene expression program could be achieved. Furthermore, the compounding need for p38α MAPK signaling provides a fine-tuning mechanism that ensures a highly regulated spatio-temporal expression pattern for these genes. Thus our findings have revealed a previously unappreciated mechanism to control the potency and function of cell fate determinants. Interestingly, unlike MyoD whose expression is restricted to cells of the muscle lineage, Six4 and Mef2d are expressed in multiple tissue types. Similarly, p38α MAPK signaling is involved in directing differentiation of multiple cell types. Thus, it is interesting to speculate that in other tissues, alternate bHLH transcriptional activators might collaborate with Six4, Mef2d and p38α MAPK to regulate cell-specific gene expression through a similar mechanism.

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