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Review

Gene Regulatory Networks and Transcriptional Mechanisms that Control Myogenesis

Margaret Buckingham[1,*] and Peter W.J. Rigby[2,*]
[1]CNRS URA 2578, Department of Developmental and Stem Cell Biology, Institut Pasteur, 75015 Paris, France
[2]Division of Cancer Biology, Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, UK
*Correspondence: margaret.buckingham@pasteur.fr (M.B.), peter.rigby@icr.ac.uk (P.W.J.R.)
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We discuss the upstream regulators of myogenesis that lead to the activation of myogenic determination genes and subsequent differentiation, focusing on the mouse model. Key upstream genes, such as *Pax3* and *Pax7*, *Six1* and *Six4*, or *Pitx2*, participate in gene regulatory networks at different sites of skeletal muscle formation. MicroRNAs also intervene, with emerging evidence for the role of other noncoding RNAs. Myogenic determination and subsequent differentiation depend on members of the MyoD family. We discuss new insights into mechanisms underlying the transcriptional activity of these factors.

Introduction

The MyoD family of myogenic regulatory factors (MRFs) controls the formation of skeletal muscle. More than 25 years ago it was shown that the members of this family of basic-helix-loop-helix (bHLH) transcription factors, when overexpressed in nonmuscle cells, will activate the myogenic program, with suppression of other cell fates and formation of differentiated muscle (see Weintraub et al., 1991). Since then the possibility of converting one cell type to another by transdifferentiation has become a major issue in the stem cell field. However, the phenomenon of myogenic conversion remains remarkable in that a single transcription factor can exert this overriding effect. We will discuss the regulatory mechanisms that underlie myogenic factor function. We will also discuss the upstream factors that direct a cell toward the skeletal muscle program, leading to activation of a gene of the *MyoD* family and subsequent formation of skeletal muscle. The focus will be on myogenesis in mammals, where skeletal muscle cell lines and the mouse model for genetic manipulation have facilitated molecular analyses.

The Formation of Skeletal Muscle

There are four MyoD family members. Compound mutations in the mouse have shown that MyoD, Myf5, and Mrf4 function as myogenic determination factors; in the absence of all three, no skeletal muscle forms. The fourth member, Myogenin, acts as a differentiation factor, as can Mrf4 and MyoD, controlling the differentiation of myoblasts into skeletal muscle fibers (see Moncaut et al., 2013). This correlates with initial observations on the greater efficiency of myogenic conversion by the three determination factors compared to Myogenin, as a result of the presence of a C-terminal domain that recruits chromatin remodelling complexes (see Fong and Tapscott, 2013).

Activation of myogenic determination genes in the embryo shows distinct temporal and spatial characteristics (see Buckingham and Mayeuf, 2012). Skeletal muscle in the trunk and limbs derives from somites that progressively form by segmentation of paraxial mesoderm on either side of the neural tube, following an anterior-posterior developmental gradient (Figure 1).

The somite is initially an epithelial ball of cells that subsequently distribute into a ventral mesenchymal sclerotome, giving rise to the bones of the vertebral column and ribs and an adjacent syndetome, a source of muscle tendons in the trunk. The dorsal part of the somite, the dermomyotome, retains an epithelial structure for longer and gives rise to dorsal dermis and all the skeletal muscles of the trunk and limbs, as well as endothelial and smooth muscle cells of blood vessels, and brown fat. Myogenesis is initiated in the somite, where *Myf5* is the first myogenic regulatory gene to be activated in the epaxial domain, adjacent to the neural tube. Subsequently this gene is activated in the opposing hypaxial domain. The closely linked *Mrf4* gene is also activated early, although at later stages it is expressed only in differentiating muscle cells. *MyoD* is transcribed after the onset of *Myf5* expression in the hypaxial and then in the epaxial dermomyotome. Myogenic cells delaminate from the dermomyotome to form the underlying differentiated muscle of the myotome, which subsequently grows and splits to form the muscles of the trunk. Cells also delaminate from the hypaxial dermomyotome to migrate to more distant locations, notably to the limbs where *Myf5* and *MyoD* are activated, leading to skeletal muscle formation. Subsequently the central dermomyotome loses its epithelial structure and myogenic progenitor cells enter the underlying myotome. These cells either activate *Myf5* and *MyoD* and differentiate or proliferate, providing a reserve cell population for muscle growth during development. Maintenance of such a progenitor population is a common feature of all muscle masses.

Skeletal myogenesis in the head also depends on the activation of myogenic determination genes (see Sambasivan et al., 2011). In this case, skeletal muscles form from cranial mesoderm, or from prechordal mesoderm in the case of the most anterior extraocular muscles. Most head muscles derive from the mesodermal core of the branchial arches, transitory structures that protrude ventrally in pairs from the pharynx, such that the muscles of mastication and facial expression derive from the first and second arches, respectively. This mesoderm also contributes to the formation of the arterial pole of the heart

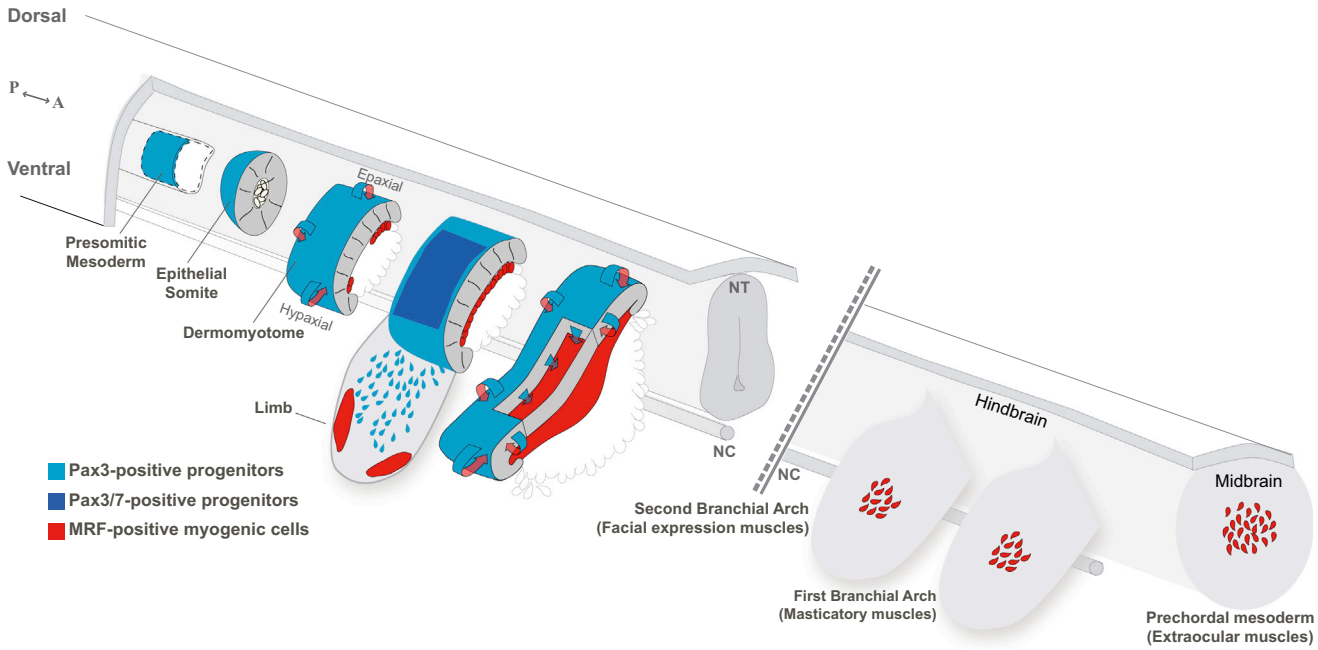

Figure 1. Schematic Representation of Somites, First and Second Branchial Arches, and Prechordal Mesoderm that Are the Sources of Skeletal Muscles, Shown for the Mouse Embryo

Somites mature following an anterior (A) to posterior (P) developmental gradient. NT, neural tube; NC, notochord.

and, although the regulation of skeletal and cardiac muscle differentiation is quite different, these progenitor cells express common upstream factors and belong to the same cell lineages. Activation of myogenesis in the head, compared to the body, therefore depends on different upstream factors and also responds differently to signaling pathways. It shows site-dependent regulation. Branchial-arch-derived muscles depend on Myf5/Mrf4/MyoD, whereas extraocular muscle formation is initiated by Myf5/Mrf4 and in their absence cannot be rescued by MyoD.

Myogenesis during muscle regeneration in the adult depends on satellite cells that are closely associated with muscle fibers. These cells probably arise from somite-derived myogenic progenitors in the body or from embryonic progenitors of head muscles. When quiescent satellite cells are activated in response to muscle damage, they proliferate and differentiate to form new muscle fibers. As in the embryo, their entry into myogenesis depends on Myf5 and MyoD (Figure 2A; see Montarras et al., 2013).

Upstream Regulators of Myogenesis

The myogenic determination factors control entry into the myogenic program, which leads to the formation of skeletal muscle. However, upstream of this obligatory step, other transcription factors direct cells toward myogenesis. Their respective roles have become clearer in the last decade, so that a regulatory network begins to emerge.

Pax3 and Pax7

The Pax family of paired domain transcription factors play key roles during tissue specification and organ development. In the context of myogenesis, Pax3 and Pax7 are important upstream regulators (see Buckingham and Relaix, 2007). Unlike the MRFs, Pax3 and Pax7 are not tissue specific, being also expressed in neurectoderm, in subdomains of the brain, in the dorsal neural tube, and in neural crest.

Pax3 Function at the Onset of Myogenesis in the Embryo

Pax3 is expressed in presomitic mesoderm and throughout the epithelial somite, before becoming restricted to the dermomyotome. It marks migrating myogenic progenitor cells that have not yet activated the myogenic determination genes and indeed the most striking feature of the *Pax3* mutant is the lack of limb muscles (see section on limb muscle progenitors). A second major feature of the *Pax3* mutant is cell death that is particularly pronounced at later stages in the hypaxial domain of the somite.

Apart from the *c-met* gene (Epstein et al., 1996), until recently very few Pax3 targets had been identified in an in vivo myogenic context. Most information was provided by analyses of cell lines derived from alveolar rhabdomyosarcomas, muscle tumors that are caused by a chromosomal translocation that results in a PAX3-FKHR(FOXO1A) or PAX7-FKHR fusion protein, acting as a strong transcriptional activator (see Robson et al., 2006). Cell death complicates loss-of-function screens in the mouse embryo, but a gain-of-function screen of Pax3-expressing cells (Lagha et al., 2010) revealed genes that are up- or down-regulated in somites and forelimbs in the presence of an allele of *Pax3* encoding a PAX3-FKHR fusion protein. These include transcription factors and components of signaling pathways known to affect myogenesis, including genes that are involved at different stages in the myogenic progression of a somitic cell (Figure 2B). In the multipotent cells of the somite, reciprocal negative regulation between *Pax3* and *Foxc2* is observed. *Foxc2*, like *Pax3*, is expressed throughout the epithelial somite.

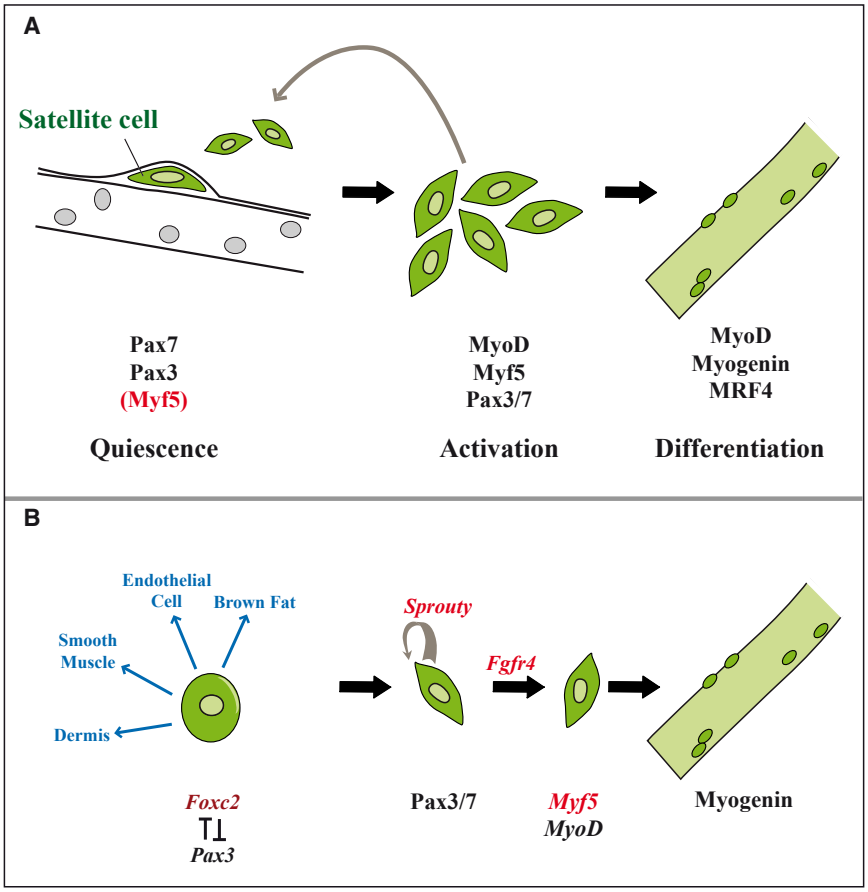

Figure 2. Progression of Muscle Progenitor Cells Toward Formation of Differentiated Skeletal Muscle

(A) The progression of adult muscle satellite cells toward new muscle fiber formation. Myf5 is shown in red in the quiescent state to indicate that transcripts are present but not the protein.

(B) The progression of somitic cells toward myogenesis, showing how Pax3 activates target genes that regulate different stages of this process. Pax3 target genes are shown in red.

Subsequently Foxc2 remains high in the sclerotome and is important for bone and cartilage formation. In the dermomyotome, expression is reduced except in the hypaxial domain. When the balance between *Pax3* and *Foxc2* expression is manipulated in Pax3-expressing cells, the myogenic cell fate is promoted by a relative increase in Pax3, whereas nonmyogenic fates of dermomyotomal cells, e.g., vascular smooth muscle or endothelial, are promoted by higher levels of Foxc2 (Lagha et al., 2009). In the chick dermomyotome, single cell labeling experiments have identified signaling pathways that influence the fate of cells derived from a single progenitor, such that BMP or Notch signaling, for example, promote vascular versus myogenic cell fates (Ben-Yair and Kalcheim, 2008). In the mouse embryo, clonal analysis also indicates multipotent Pax3-positive progenitors (Esner et al., 2006) for these fates. Once Pax3-positive myogenic cells have left the dermomyotome and entered the forming muscle mass (Figure 1), a critical cellular equilibrium has to be maintained between self-renewal of the progenitor cell pool and myogenesis. This basic stem cell requirement for tissue growth depends on the control exerted by signaling pathways (see Buckingham and Mayeuf, 2012). Notch signaling, for example, promotes self-renewal so that when the Notch pathway is mutated the myogenic progenitor pool is depleted by excessive differentiation, leading to a later reduction in muscle mass. FGF signaling is also implicated in maintaining this balance. Pax3 intervenes at this level by directly controlling a myogenic enhancer element 3′ of the *Fgfr4* gene and also by affecting expression of *Sprouty* genes that encode intracellular inhibitors of tyrosine kinase receptor signaling.

At the point of entry into the myogenic program, Pax3 regulates enhancer elements of *Myf5*. The transcription of *Myf5* at different sites in the embryo is controlled by a large number of enhancers distributed over more than 100 kb upstream of the gene (see Moncaut et al., 2013), permitting precise spatio-temporal regulation of the onset of myogenesis. The multitude of Myf5 enhancers that regulate expression in the somites and the arches are exquisitely specific for their target gene: they do not activate the closely linked Mrf4 gene. This is achieved by a novel mechanism that depends on transcription balancing sequences (TRABSs), one of which is located in the interval defined as the proximal arch enhancer. TRABSs act to regulate the equilibria between the enhancers and the promoters of Myf5 and Mrf4 (Carvajal et al., 2008). Early activation in the epaxial dermomyotome depends on canonical Wnt signaling from the neural tube and is modulated by Sonic Hedgehog signaling from the midline, targeting the early epaxial enhancer (EEE) through Tcf and Gli binding sites. Pax3 does not directly target the EEE although it can indirectly affect it through the Dmrt1 transcription factor, because *Dmrt1* is a direct Pax3 target (see Buckingham and Mayeuf, 2012). Two enhancer elements that regulate *Myf5* expression in the hypaxial somite/myotome (at −110 kb) and in the limbs (at −57.5 kb) have been shown to be direct Pax3 targets and their activity is Pax3 dependent. Activity of these enhancers is also directly regulated by signaling pathways, such that the −110 enhancer depends on a Tead binding site, a read-out of the Hippo pathway, and the −57.5 enhancer on essential Gli binding sites that respond to Sonic Hedgehog signaling from the ZPA in the ventral domain of the limb, showing how signals as well as upstream myogenic regulators control the onset of myogenesis. The examples of Pax3 targets cited here illustrate how this key factor orchestrates different steps in the progression of a multipotent somitic cell to a tissue-specific myoblast at the onset of myogenesis (Figure 2B).

Initial activation of *MyoD*, which takes place after *Myf5,* depends genetically on *Myf5* and also on *Pax3* because in *Myf5(Mrf4)/Pax3* compound mutants, no skeletal muscle forms in the trunk and limbs (Tajbakhsh et al., 1997). Embryonic expression of *MyoD* depends on an enhancer at −20 kb (CE) from the gene (see Tapscott, 2005), which is not a direct Pax3 target, although a Pax binding site in the *MyoD* promoter is targeted by Pax7 in postnatal muscle cells (Hu et al., 2008).

The Role of Pax7 during Fetal and Postnatal Myogenesis

Whereas Pax3 is expressed in all myogenic progenitor cells in the embryo, Pax7 is mainly present in the central domain of the dermomyotome and in the absence of Pax3 it is this domain that survives. These Pax3/Pax7-positive cells provide the self-renewing reserve cell population for muscle growth (see Buckingham, 2006). In double *Pax3/Pax7* mutants, these cells fail to activate *Myf5* or *MyoD* and assume other cell fates or die. There is a major muscle deficit, with the presence of only those muscles derived from the primary myotome that formed as a result of early activation of *Myf5* through the epaxial enhancer. *Pax3* transcription is downregulated in fetal muscle when Pax7 becomes the dominant factor in all myogenic progenitor cells. In the limb, *Pax7* is initially coexpressed with *Pax3* and genetic tracing experiments show that all later Pax7-positive cells in the fetal limb are derived from cells that had expressed *Pax3* (Hutcheson et al., 2009). Activation of fetal-specific muscle genes depends on the Nfix transcription factor, where *Nfix* is a potential Pax7 target (Messina et al., 2010). Postnatal and adult satellite cells are marked by Pax7 expression, with continuing transcription of *Pax3* in trunk muscles such as the diaphragm and some limb muscles (see Montarras et al., 2013). Prior to birth, Pax7 is not essential for myogenesis, presumably because Pax3 can compensate. After birth, on the other hand, *Pax7* mutants lose their satellite cells and Pax3 cannot compensate even in trunk muscles such as the diaphragm, perhaps because the protein is present at too low a level or because of divergent Pax3 and Pax7 functions by this stage (Soleimani et al., 2012a). Pax7-negative satellite cells can initiate differentiation, probably due to transcription of *Myf5* in an increasing number of these cells from the perinatal period. Consistent with a role for Pax7 in the initiation of *MyoD* but not *Myf5* transcription in most satellite cells in culture, introduction of dominant-negative Pax7 specifically abolishes MyoD (Relaix et al., 2006) but not Myf5 expression or satellite cell differentiation. The role of Pax7 in adult satellite cells has been controversial. A first report on conditional *Pax7* mutants indicated that the satellite cell population was still present and that muscle regeneration could take place, even in the absence of both Pax7 and Pax3 (Lepper et al., 2009). Since then this view has been modified and in a more extensive study muscle regeneration was shown to be severely impaired when *Pax7* ablation is attained in most satellite cells, preventing repopulation of the satellite cell pool (Günther et al., 2013; von Maltzahn et al., 2013). In this adult situation the satellite cell pool is not maintained, not due to cell death but probably because of premature differentiation at the expense of proliferation (Günther et al., 2013). Pax3/Pax7 are normally downregulated prior to activation of *Myogenin,* cell cycle exit, and differentiation. Artificial maintenance of their expression in myoblasts has been reported to retard differentiation (Crist et al., 2012; Olguin and Olwin, 2004). In this context, it has been proposed that Pax3 can promote satellite cell proliferation (Conboy and Rando, 2002). As in the embryo, Pax intervention in the balance between self-renewal and differentiation is probably critical.

Very little is known about Pax7 targets in satellite cells during muscle regeneration. In addition to Pax7 activation of *MyoD* through a site in the promoter (Hu et al., 2008), the −110 kb element upstream of *Myf5* has now been shown to be active in satellite cells where it binds Pax7 (Soleimani et al., 2012a). Pax3/7 has been shown to directly activate *Id*3, which encodes a HLH inhibitor of myogenic factor activity, potentially preventing, together with Id2, the onset of myogenesis in quiescent satellite cells (Kumar et al., 2009). New insight into potential Pax7 targets in satellite cells comes from genome-wide ChIP-seq and transcriptome analyses carried out on primary myoblasts derived from cultured satellite cells, in which a tagged Pax7 protein was expressed (Soleimani et al., 2012a). This suggests that Pax7 targets many genes implicated in satellite cell function, including genes involved in cell growth, cell adhesion, and signaling pathways, whereas it represses genes involved in differentiation.

Transcriptional Mechanisms

In this analysis, tagged Pax3 was also expressed and shown to bind to fewer sites than Pax7. Both factors bind through a paired domain or paired and homeodomains, but Pax7 (not Pax3) also binds with high affinity through the homeodomain only. This therefore points to significant differences between the functions of the factors. Pax3 does not rescue the postnatal phenotypes of the *Pax7* mutant in muscles where both proteins are present in satellite cells. However, during myogenesis in the embryo, Pax7, when it is coexpressed with Pax3, can compensate, as in the Pax3/Pax7 progenitor cell population derived from the central dermomyotome. Introduction of a Pax7 coding sequence into an allele of *Pax3* showed that Pax7 can replace Pax3 function during myogenesis in the trunk and also partially in the limbs (see Buckingham and Relaix, 2007). Functional differences between Pax3/Pax7 in postnatal versus prenatal myogenesis may reflect post-transcriptional modifications of the proteins and also association with different cofactors. Pax3 activity requires phosphorylation (Miller et al., 2008) and interference with this affects Pax3 function in the hypaxial somite (Brunelli et al., 2007). During embryonic myogenesis, Pax3 functions as a transcriptional activator (see Buckingham and Relaix, 2007); however, like other Pax proteins, it is a poor activator on its own, indicating the probable importance of coactivators. To date these have not been identified at sites of myogenesis in vivo. Pax7 has been shown to interact with the histone methyl transferase complex Wdr5-Ash2L-MLL2, which directs activating H3K4 histone modifications (McKinnell et al., 2008) in myoblasts from postnatal muscle. *Pax7* mutant satellite cells show reduced heterochromatin condensation (Günther et al., 2013), pointing to a role in chromatin organization, also recently suggested for Pax3 (Bulut-Karslioglu et al., 2012). Recent research begins to provide some insight, but mechanistically much remains to be understood about the function of Pax3 and Pax7 as transcription factors.

Six1 and Six4, with Eya1 and Eya2 Cofactors

Six homeodomain transcription factors, with Eya and Dach cofactors (Kawakami et al., 2000), also play an important

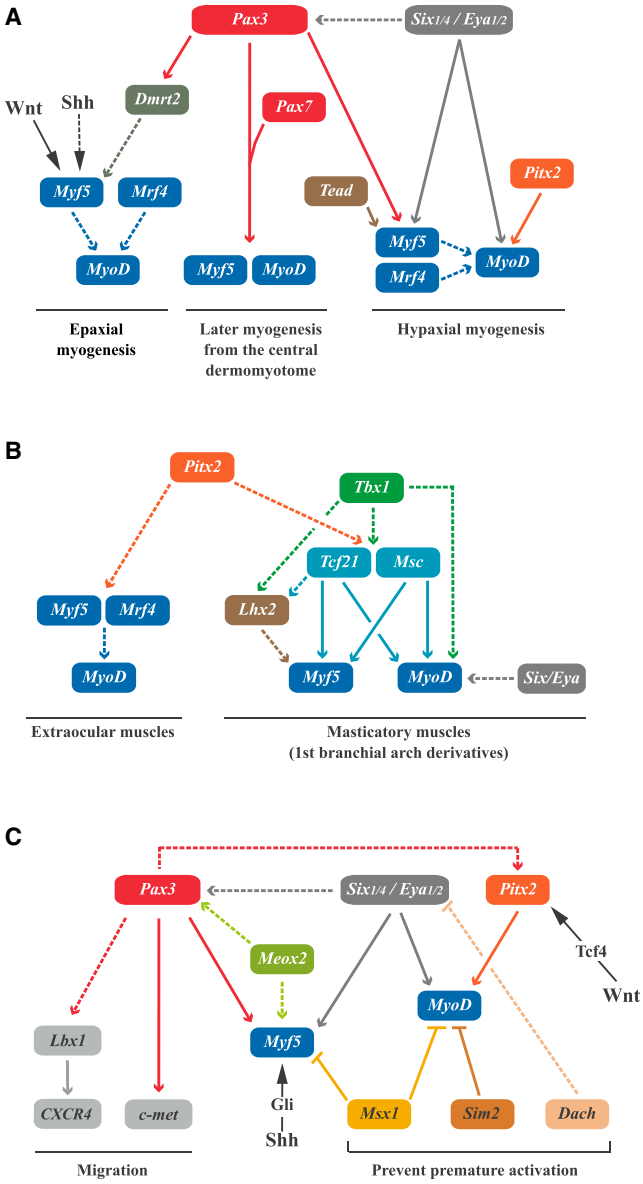

Figure 3. Gene Regulatory Networks that Govern Myogenesis
Shown in the trunk (A), the head (B), and in cells that migrate from the hypaxial somite shown here for the forelimb (C).

upstream role in myogenesis, which is linked to that of Pax3 (Figure 3). Eya factors act as phosphatases and it is proposed that this activity inhibits Dach corepressor function. Eya function also involves recruitment of coactivators such as CBP to the Six complex (Jemc and Rebay, 2007).

Functions of Six1/4 and Eya1/2 as Upstream Regulators of Myogenesis in the Embryo

The first indication of an upstream function in myogenesis came from experiments in the chick embryo where ectopic expression of Six1 and Eya resulted in activation of *Pax3* and myogenic regulatory genes (Heanue et al., 1999). Since then, mutant analysis in the mouse has provided insight into the complex roles of Six and Eya in activation of the myogenic program. In the mouse, *Six1*, *Six4*, *Eya1*, and *Eya2* are expressed in the dermomyotome and subsequently in Pax3-positive myogenic progenitors. Unlike Pax3 and Pax7, these factors are also present in differentiated skeletal muscle. In the dermomyotome, *Eya1* and *Eya2* are mainly expressed in the epaxial and hypaxial domains, after the initial onset of epaxial myogenesis. The critical role of Six/Eya in myogenesis is revealed by the phenotypes of *Six1*/*Six4* and *Eya1*/*Eya2* double mutants (Grifone et al., 2005, 2007), which are more severe than in the single *Six1* mutant, with loss of all muscles derived from the hypaxial dermomyotome, including limb and many trunk muscles. Although Six/Eya is not active in the central dermomyotome, the myogenic contribution from Pax3/Pax7 progenitors derived from this domain is also compromised, as indicated by later fetal phenotypes, suggesting an indirect effect due to the absence of hypaxial muscles. Epaxial myogenesis leading to the formation of back muscles takes place. This probably reflects its early onset, prior to a functional effect of Six regulation. Furthermore, there is no evidence of a requirement for Six sites in the EEE of *Myf5*. Myogenesis arising from posterior somites is not affected in the mutant, reflecting the absence of Six/Eya activity, as indicated by a transgene reporter (Grifone et al., 2007). In these *Eya* or *Six* double mutants, *Six1/4* or *Eya1/2* expression, respectively, is not impaired, indicating that they are not interdependent.

There are two striking features of these double mutants. First, *Pax3* expression is lost in the hypaxial dermomyotome, which would account for the absence of progenitor cell migration and cell death. Surviving cells do not migrate but mislocate and acquire other cell fates, reminiscent of the *Lbx1* mutant (see section on limb muscle progenitors). Pax3 targets in the hypaxial dermomyotome, such as *Lbx1* or *c-met*, are not expressed, possibly also reflecting a direct effect of Six1/4, since this phenotype is observed prior to the major loss of Pax3 and cell death. Downregulation of *Pax3* may be effected through a hypaxial enhancer upstream of the promoter, which can direct transgene expression to this domain in anterior somites (Brown et al., 2005) and binds Six1 in vivo (Grifone et al., 2007).

The second striking feature of the double mutants is the pronounced downregulation of myogenic regulatory genes from the time when the Six/Eya complex would normally be active. This is particularly pronounced for *Mrf4,* which is no longer expressed at sites of hypaxial myogenesis in the trunk, and is also seen for *Myf5* and *MyoD*. Six/Eya directly regulates enhancer elements of the *Myf5* and *MyoD* genes. For *Myf5* this has been demonstrated for the −57.5 enhancer that controls *Myf5* activation in the limbs and mature hypaxial dermomyotome (Giordani et al., 2007). Thus, Six and Pax are required together for correct expression of *Myf5* directed by the −57.5 enhancer. *MyoD* expression is controlled by an embryonic enhancer (CE) at −20 kb and a second distal enhancer at −6 kb (DRR) (see Tapscott, 2005). Both of these regulatory elements contain sites that bind Six1 and Six4 and interact with Six/Eya complexes in vivo. Mutation of these sites results in almost complete abolition of expression of a transgene controlled by the two enhancers and the proximal promoter, demonstrating direct regulation of *MyoD* by the Six/Eya complex (Relaix et al., 2013). This is observed not only in the trunk and limbs, but also in head muscles, where *Six* genes are expressed at sites of myogenesis. However *Six1*/*Six4* double mutants did not show any phenotype in these muscles, probably due to compensation by Six2.

In the genetic hierarchy that regulates the onset of myogenesis (Figure 3), Six activation of *MyoD* is an important facet. *Six1/Six4/Myf5(Mrf4)* compound mutants do not activate *MyoD* and do not form skeletal muscles in the trunk and limbs (Relaix et al., 2013). This resembles the phenotype of *Pax3/Myf5(Mrf4)* mutants (Tajbakhsh et al., 1997). It is perhaps surprising that in the *Pax3/Myf5(Mrf4)* compound mutants, later activation of *MyoD* by Six/Eya, or indeed by Pax7, does not take place in the absence of Pax3. This points to the importance of the early myotome as a scaffold and source of signaling molecules for subsequent myogenesis, as well as the role of Pax3 in the survival of those myogenic progenitors that do not express Pax7.

Six genes are also expressed in adult satellite cells, where Six1 plays a role in regulating the regenerative capacity of these cells and their proliferation (Le Grand et al., 2012; Yajima et al., 2010), properties that are also Pax7 dependent.

Regulation of Skeletal Muscle Differentiation by Six/Eya

The proximal regulatory region of *Myogenin* is also directly controlled by Six factors (Spitz et al., 1998) and again the double *Six1/Six4* and *Eya1/Eya2* mutant phenotypes indicate Six/Eya regulation of this myogenic differentiation gene. Transcription of *Myogenin* also depends on other elements (Cheng et al., 1993; Yee and Rigby, 1993), which probably accounts for Six-independent expression of *Myogenin,* seen at remaining sites of muscle differentiation.

Six/Eya also controls downstream muscle genes, notably those associated with a fast glycolytic muscle phenotype that are downregulated in *Six1/Six4* double mutants (Richard et al., 2011). *Sox6*, involved in suppressing the slow muscle phenotype in the mouse embryo, is not expressed in the *Six1/Six4* double mutant. Six1 and Six4 bind to and transactivate regulatory regions of fast muscle genes (Niro et al., 2010). Six1 and Eya1 are enriched in fast glycolytic fibers of adult muscle and their forced expression in slow oxidative fibers will convert them to a fast glycolytic phenotype. These effects on the activation of downstream transcription factor and muscle genes distinguish Six/Eya from Pax regulation of myogenesis. Pax7 may directly repress genes required for differentiation (Soleimani et al., 2012a), but the main role of Pax3 and Pax7 is in controlling upstream events leading to myogenesis. Pax3 is active in the somite prior to Six/Eya intervention and has more wide-reaching effects at the onset of myogensis. However, Six/Eya play a major role in the onset of hypaxial myogensis, both directly through activation of myogenic determination genes and also indirectly through control of *Pax3* (Figure 3A).

Pitx2

The three *Pitx* genes present in vertebrates encode a family of paired-related homeodomain transcription factors. They were first identified as regulators of pituitary development and play an important role in the formation of multiple organs, in craniofacial development, and in the late read-out of left/right signaling (Gage et al., 1999).

Pitx Function during Myogenesis in the Trunk and Limbs

During myogenesis in the embryo, *Pitx2* is expressed in myogenic progenitor cells whereas *Pitx3* is expressed in differentiating muscle where it is replaced by Pitx2 in the *Pitx3* mutant (L'Honoré et al., 2007). In the absence of Pitx2 (L'honoré et al., 2010), the onset of myogenesis in the limb is affected and there are also minor myogenic defects in the somite. These are due to downregulation of *MyoD*, which is compensated by Myf5. Pitx2 regulates the CE at −20 kb that activates *MyoD* in the limb. In the somite, Pitx2 also directly controls *MyoD* transcription through sites in the CE enhancer and a site adjacent to the promoter. The DRR at −6 kb is also implicated, where Pitx2 may act in conjunction with SRF which directly regulates the DRR. In *Myf5(Mrf4)/Pitx2* compound mutants, skeletal muscle does not form. This phenotype is similar to that of the *Myf5(Mrf4)/Pax3* compound mutant, suggesting that *Pitx2* lies genetically downstream of *Pax3* (Figure 3A). In gain-of-function screens for Pax3 targets in myogenic progenitors in the embryo, *Pitx2* was upregulated, consistent with it being a Pax3 target (Lagha et al., 2010). In the *Pitx2* mutant, there are fewer proliferating myogenic cells in the somite, potentially reflecting a role in mediating proliferation (Kioussi et al., 2002).

Pitx2 and Other Factors that Control the Onset of Myogenesis in Head Muscles

Pax3 is not expressed at sites of head muscle formation and it is Pitx2 that plays a major role as an upstream regulator of craniofacial myogenesis (Figure 3B; see Sambasivan et al., 2011). Pax7 is expressed later, together with Pitx2, and marks satellite cells, as in the trunk and limbs. Extraocular muscles are absent in *Pitx2* mutant embryos where extensive cell death takes place in the premyogenic mesoderm. By a conditional mesoderm deletion of *Pitx2*, activated after the critical period of cell survival, downregulation of *Myf5* and *Mrf4,* which control the onset of myogenesis in these muscles, is observed and *MyoD* is not expressed (Zacharias et al., 2011). Experiments in cell cultures suggest that Pitx2 directly activates *Myf5* as well as *MyoD* through sites in the promoter region. At the onset of extraocular muscle formation, Pitx2 therefore regulates both progenitor cell survival and myogenic specification, assuming the role of Pax3 at sites of myogenesis in the body. Comparison of myogenic cells from extraocular and limb muscles indicates that the former express higher levels of Pitx2, required for their proliferation and differentiation, and that this high level is maintained in aging and in dystrophic muscles. This may be related to the remarkable sparing of these muscles in muscular dystrophies (Hebert et al., 2013). Recent transcriptome profiling in postnatal extraocular muscles of conditional *Pitx2* mutants suggests that Pitx2 may be important for maintaining the expression of downstream muscle genes that characterize the extraocular phenotype (Zhou et al., 2012).

The formation of masticatory muscles that derive from the first branchial arch is also defective in *Pitx2* mutants (see Sambasivan et al., 2011). *Pitx2* is expressed in ectoderm as well as mesoderm of the first arch, but a conditional deletion targeted to the mesoderm demonstrates that the survival and growth of myogenic progenitors are compromised and myogenic determination genes are not activated.

The T-box transcription factor Tbx1 is another regulator of myogenic progenitors in the first branchial arch. In *Tbx1* mutants the more posterior arches are lost, but the first arch is maintained. Masticatory muscles derived from the first arch are hypoplastic. Again, conditional mutants directed to mesoderm indicate that this is a direct effect rather than one that is mediated by endodermal or ectodermal expression of *Tbx1*. Since Tbx1 directly activates *Fgf8* and *Fgf10*, myogenic defects may be

partly due to a negative effect on the proliferation of the progenitor population, which depends on FGF signaling. However, in *Tbx1/Myf5* double mutants, all muscles derived from the first branchial arch are absent, suggesting that these genes act upstream of *MyoD.* It had been suggested that *Tbx1* and *Pitx2* may regulate each other, but in the *Pitx2* mutant, *Tbx1* continues to be expressed (Dong et al., 2006).

Tbx1 and Pitx2 are also present in cardiac progenitor cells, marked by *Islet1* expression. In this anterior region of the embryo, the distinction between paraxial mesoderm that gives rise to skeletal muscle and splanchnic mesoderm of the second heart field that is the source of myocardial cells is blurred. Retrospective clonal analysis shows that a common progenitor gives rise to branchial-arch-derived skeletal muscles and to the arterial pole of the heart (Lescroart et al., 2010). In the mesodermal core of the branchial arches, progenitors for both muscle cell types are present, with myogenic progenitors that will activate myogenic determination genes located more proximally. Genetic tracing with an *Islet1-Cre* shows labeling of facial expression muscles and a subset of masticatory muscles. *Islet1* mutants die before head muscle formation but overexpression of Islet1 represses myogenic differentiation (see Tzahor and Evans, 2011).

Two bHLH transcription factors, Msc (MyoR) and Tcf21 (Capsulin), are expressed in myogenic progenitors in the arches, prior to activation of the myogenic regulatory genes (see Sambasivan et al., 2011). In *Msc/Tcf21* double mutants, major masticatory muscles are lost and *Myf5* is downregulated in myogenic cells in both arches. *Tbx1* is upregulated in the *Msc* mutant when Myf5 and MyoD levels are reduced, suggesting that cells remain in a progenitor state. Multiple *Myf5* regulatory elements direct transcription of this myogenic regulatory gene in the branchial arches. The *Myf5* proximal arch element, which is important for early expression, binds Msc and Tcf21 in vivo and these binding sites are necessary for the correct expression of *Myf5* in a subset of cells. The DRR at −6 kb and the promoter region of *MyoD* also bind Msc and Tcf21. The implication is that Msc and Tcf21 function as transcriptional activators for *Myf5* and *MyoD,* controlling their levels of expression and thus regulating myogenic determination in the arches (Moncaut et al., 2012). These factors can also potentially interfere with myogenic activation by MRFs and in a recent ChIP-seq study, Msc binding sites in the genome have been shown to overlap with those for MyoD (MacQuarrie et al., 2013). It is not clear how *Msc* and *Tcf21* are regulated in the arches, but Pitx2 and Tbx1 are potential candidates.

The transcription factor Lhx2 has been identified as a component in the hierarchy regulating myogenesis in the branchial arches (Figure 3B). *Lhx2* lies genetically downstream of *Tbx1, Pitx2*, and *Tcf21* and upstream of *Myf5.* In vivo ChIP experiments suggest that *Lhx2* may be directly regulated by Tbx1, Pitx2, and Tcf21. *Lhx2* mutants have defects in branchial arch specification of myogenic cells and head muscle patterning, whereas *Tbx1/Lhx2* double mutants lack branchial arch muscles (Harel et al., 2012).

Lbx1, Msx1, Sim1, and Meox2 in Limb Muscle Progenitors

Myogenic progenitor cells that migrate from the hypaxial somite to form more distant muscles such as those in the limb are regulated by Pax3 but also by an additional gene hierarchy (Figure 3C) implicated in migration and in the avoidance of premature myogenesis in migrating cells.

The Pax3 target *c-met* encodes a tyrosine kinase receptor required for delamination of migratory cells. Interaction with its ligand, HGF, present in the adjacent mesenchyme, is also important for guiding cell migration (see Birchmeier and Brohmann, 2000). This regulation is essential for limb progenitors and also for other migrating myogenic cells such as those that form the diaphragm. The homeodomain factor Lbx1 controls the migration of myogenic progenitor cells through its activation of *CXCR4*, which encodes the receptor for SDFI (Vasyutina et al., 2005). In *Lbx1* mutants, muscles in the hindlimbs and dorsal forelimbs are absent with mislocation of Pax3-positive cells adjacent to the somites (Schäfer and Braun, 1999). *Lbx1* is a potential Pax3 target (Lagha et al., 2010); however, *Lbx1* is not expressed at all axial levels, but in the hypaxial domain of somites that give rise to migrating cells, as at limb level. Hox factors intervene in the activation of *Lbx1* (Alvares et al., 2003), thus providing an indication of how the *Hox* code influences myogenesis on the anterior/posterior axis. *Meox2*, together with *Meox1*, is expressed in somites all along the axis. Meox2 continues to be present in migrating myogenic progenitors. It does not appear to affect migration, but in the *Meox2* mutant certain limb muscles are lost, notably in the forelimb. Once progenitor cells reach the limb, Pax3 expression is downregulated and there is a delay in *Myf5* activation (Mankoo et al., 1999), in keeping with the role of Meox homeodomain proteins as transcriptional activators.

An important question for myogenesis is why progenitor cells expressing factors such as Pax3 or Six/Eya do not immediately activate downstream myogenic factor genes and differentiate. Under some conditions this is probably due to the modulating effect of signaling pathways such as Notch or FGF. It may also reflect the presence of corepressors such as Dach, present in the Six-positive cells that migrate to the limb (Heanue et al., 1999). However, in the context of the limb, there is also evidence for the action of transcriptional repressors such as the homeodomain protein Msx1 or the bHLH-PAS-domain factor Sim2. *Msx1* is expressed in the hypaxial dermomyotome and in migrating Pax3-positive cells, at forelimb level (Houzelstein et al., 1999). *Msx1* in this context is directly regulated by Tcf4 (Miller et al., 2007), suggesting a role in limb muscle patterning that is under the control of canonical Wnt signaling (Hutcheson et al., 2009). *Msx1* is downregulated prior to activation of *Myf5.* Forced expression of Msx1 antagonizes differentiation in muscle cell lines (Song et al., 1992) and prevents myogenic conversion of fibroblasts by MyoD, with recruitment of the linker histone H1B that represses *MyoD* transcription (see Tapscott, 2005). More recently Msx1 has been shown to recruit the repressive Polycomb complex to the −20 kb enhancer of *MyoD* and to the *Myf5* −57.5 kb regulatory region that controls the onset of expression in the limbs (Wang et al., 2011). This is accompanied by a redistribution of H3K27me3 repressive marks due to recruitment of G9a methyltransferase (Wang and Abate-Shen, 2012). The developmental significance of these observations is suggested by *Myf5* upregulation in the forelimbs of *Msx1* mutant embryos (Wang et al., 2011). Sim2 is expressed in muscle progenitors in the limb, prior to their entry into the myogenic

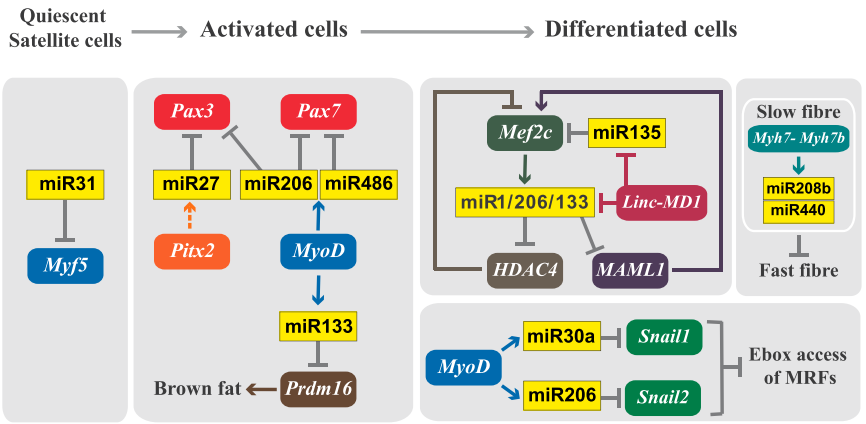

Figure 4. MicroRNA Regulatory Networks in Satellite Cells as They Progress toward Differentiation, when Cultured or during Regeneration of Adult Muscle after Injury
Abbreviation: miR, microRNA.

program. It is recruited to the −20 kb *MyoD* enhancer and represses *MyoD* expression, thereby also preventing premature activation of myogenesis in Pax3-positive progenitors (Havis et al., 2012). Its expression principally in ventral muscle masses, indirectly under the repressive action of the dorsalizing factor Lmx1b, also provides insight into muscle patterning.

MicroRNAs

Transcriptional regulation of the onset of skeletal muscle formation is modified by posttranscriptional mechanisms that affect the presence and function of the transcription factors concerned. MicroRNAs (miRNAs), which impact mRNA stability and translation through interaction with specific sites in the 3′ UTR, have emerged as important components of the myogenic regulatory network (Figure 4; see Gagan et al., 2012; Goljanek-Whysall et al., 2012). Pax3 and Pax7 mRNAs are both targeted by miR206 and in addition they are targeted by miR27 and by miR486 and miR1, respectively. These miRNAs are present in myogenic cells that express MyoD and Myf5 and play a role in the downregulation of the Pax factors when the cells differentiate, as well as affecting their survival and/or proliferation. Their role in modulating the onset of differentiation is illustrated by a recent report on miR206 that promotes muscle regeneration (Liu et al., 2012). The *miR206* gene is directly activated by MyoD and Pitx2 has been reported to regulate the expression of *miR27* in cultured muscle cells. Susceptibility to miRNA regulation depends on the 3′ UTR sequence, which may vary in different splice forms of the mRNA. In the case of Pax3, in different populations of myogenic progenitors, alternative polyadenylation results in transcripts that have longer or shorter 3′ UTRs, where only the longer form has miR206 and miR27 sites (Boutet et al., 2012), thus affecting Pax3 regulation. The mRNA for Msc is targeted by miR378, which is under MyoD control.

The mRNA for Myf5 is targeted by miR31, preventing precocious activation of myogenesis in the epaxial dermomyotome or inappropriate and potentially dangerous myogenesis in regions of the central nervous system where *Myf5* is transcribed in the mouse embryo. It also prevents accumulation of Myf5 protein and consequent activation of myogenesis in quiescent satellite cells, many of which transcribe *Myf5* and in which the mRNA is sequestered in RNP particles with miR31 (Crist et al., 2012). Experiments, principally on muscle satellite cells, have revealed miRNA control of proliferation via mRNAs coding for regulators of the cell cycle, thus promoting satellite cell quiescence or cell cycle withdrawal prior to differentiation. miRNAs also fine-tune signaling pathways that play a role in myogenesis, such as the IGF pathway, or regulate miRNA genes as shown for TGFβ that represses miR1/206. In addition to the MRFs, Mef2 factors are also key regulators of muscle genes at the onset of differentiation. Mef2c, like MyoD, activates *miR1/206/133* gene clusters. Again, feedback circuits operate such that miR1 controls the level of HDAC4 that represses *Mef2C,* whereas miR133 targets the mRNA of MAML1, which is a coactivator of Mef2. A new level of complexity emerges with the demonstration that the preRNA for miR133 also encodes a competing endogenous (ce) RNA, linc-MD1, which binds to and sequesters miR133 and also miR135, which targets the mRNA for Mef2c (Cesana et al., 2011).

MicroRNAs also regulate the muscle phenotype. miRNAs encoded by introns of slow myosin genes modulate the expression of factors that control slow versus fast fiber type specification. Recently it has been shown that miRNAs can play an upstream role in cell fate determination. Adult satellite cells normally enter myogenesis and form muscle fibers. However, they transcribe *Prdm16* that encodes a transcriptional regulator of brown fat, derived from Pax3-expressing cells in the somite. miR133 targets the 3′ UTR of Prdm16 mRNA and prevents accumulation of Prdm16. In the absence of miR133 activity, satellite cells give rise to brown adipocytes and it is proposed that downregulation of miR133 on cold exposure permits satellite cell conversion to thermogenic brown fat cells (Yin et al., 2013).

Thus, posttranscriptional control of myogenesis by miRs is an important facet of the regulatory network (Figure 4). We have discussed examples that affect upstream factors, but the miRNAs mentioned have multiple targets and many other miRNAs are present in muscle cells, so that their modulating influence is likely to be highly complex. In addition to other classic mechanisms that impact mRNA or protein levels and function, other classes of noncoding RNAs are just beginning to emerge as additional layers that confer transcriptional or posttranscriptional regulation.

Mechanisms Underlying MRF Function

Once the cascade of MRFs has been activated, these bHLH transcription factors act as obligate heterodimers with the ubiquitously expressed E proteins to activate the terminal differentiation program by regulating the transcription of many genes including those encoding the contractile proteins and muscle-specific enzymes, as well as a number of miRNAs (Figure 4). It has been known for many years that MRFs do this, at least in part, by binding to E-boxes in the promoters and enhancers of

their target genes and by recruiting coregulators and RNA polymerase II (PolII), and that they often act in concert with other transcription factors, for example Mef2. Most of this knowledge is derived from experiments on single genes. The advent of genome-wide technologies has allowed these issues to be readdressed and led to a number of important new mechanistic insights.

Much of this work has used as a model the in vitro differentiation of the C2 muscle cell line and analyzed the transcriptome, protein-DNA interactions, and epigenetic modifications in proliferating myoblasts and in nondividing myotubes derived from them. C2 cells are generally thought to be related to satellite cells that are derived from muscle progenitors in the embryo: the generalizability of the conclusions that we will discuss has not been extensively tested. A second widely used approach has been to introduce one of the MRFs, almost always MyoD, into a naive cell, usually mouse embryonic fibroblasts (MEFs), and then to study what happens as these cells enter the myogenic program and differentiate into myotubes. Microarray experiments identified the genes that are regulated, in multiple waves, during this transition and indicated that MyoD acts at all stages of the differentiation process via a feed-forward loop (see Tapscott, 2005). The assumption was, of course, that MyoD was bound to the control elements of the genes being regulated and ChIP-seq technologies have now been used to test the assumption, with some surprising results.

How Many MyoD Binding Sites Are There?

Cao et al. (2010) found that the protein bound to the control elements of genes regulated during differentiation but also to a very large number of sites not obviously associated with such regulated genes. The number of sites (23k in C2 myoblasts, 26k in myotubes) is much higher than expected and most of the sites are the same in the two cell types. The function of the additional binding sites is not clear. Most of them are inactive in a transfection assay for enhancer function, so they could reflect an unknown function of MyoD or simply the fact that the protein will bind to all E-boxes with some affinity. Mousavi et al. (2013) have performed a similar analysis and also detected an unexpectedly high number of sites (18k in myoblasts, 40k in myotubes). Furthermore, they found 35k Myogenin (MyoG) sites in myotubes, the majority of which overlap with MyoD sites.

Soleimani et al. (2012b) did the experiment differently, using retroviral transduction to introduce a TAP-tagged MyoD into myoblasts derived from cultured satellite cells and then the tag to recover the protein and its bound DNA. They found a much smaller number of sites (1.4k in myoblasts, 9.3k in myotubes) and observed a significant difference between the cell types. The exogenous protein is expressed at a higher level than the endogenous one but that would be expected to lead to more occupied sites, not less, and Yao et al. (2013) have shown that 4-fold overexpression of MyoD does not lead to a significant increase in the number of bound sites. Apart from analysis of endogenous protein versus tagged exogenous MyoD on a wild-type background, the reasons for this interestingly different result may lie in the data handling and analysis pipelines; no doubt future work will clarify the situation.

Blum et al. (2012) have taken a different approach, identifying enhancers in C2 myoblasts and myotubes on the basis of histone marks and then asking whether PolII, transcription factors, and coactivators are bound to them. The identified enhancers that are close to genes are linked to those expressed in the relevant cell type but rather few of these condition-specific "enhancers" are associated with transcripts (eRNAs). Perhaps the marks analyzed are found on elements with other functions? Comparisons with the data of Cao et al. (2010) show that MyoD binding to enhancers correlates with the binding of PolII and p300; in *MyoD*$^{-/-}$ cells these proteins are not present and there is a significant decrease in transcription across the enhancers. Reintroduction of MyoD in null myoblasts causes a restoration of PolII binding and H3K4 monomethylation but not of H3K27 acetylation, whereas in myotubes acetylation is restored as well. Such re-expression experiments are an important way to examine cause and effect relationships.

How Does MyoD Find the Right Sites?

What is clear is that these and related data have illuminated a number of mechanistic questions that have preoccupied the field for many years. Just as MyoD regulates muscle-specific genes, the closely related transcription factor NeuroD drives a neuronal program. How is it that transcriptional activation by two very similar proteins can lead to such different outcomes? Fong et al. (2012) introduced NeuroD into P19 cells, a pluripotential mouse embryonal carcinoma cell line, and thus converted them into neurons, and MyoD into MEFs. NeuroD can not convert MEFs into neurons and MyoD can not convert P19s into muscle. The data again show an unexpectedly high number of binding sites, for both factors, but they reveal a very interesting specificity. Both proteins bind to the CAGCTG E-box but they each also bind to a particular E-box (CAGATG for NeuroD and CAGGTG for MyoD), and this binding is translated into differential function. Binding to the specific E-box sequences leads to activation of adjacent genes whereas binding to the common sequence correlates with regional epigenetic modification. The binding of both MyoD and NeuroD is constrained by chromatin accessibility and thus which sites are open is determined epigenetically in a lineage-specific fashion; sequence specificity then dictates which factor binds where and thus which genes are subsequently activated.

A Novel Mechanism of Transactivation

It has become apparent in recent years that a very high proportion of the genome is transcribed into a variety of classes of RNA that do not encode proteins. Of particular interest in the present context are eRNAs, which may or may not be polyadenylated and are transcribed from active enhancers. These eRNAs may be functional, or they may simply be a by-product of the fact that PolII is loaded onto active enhancers. Mousavi et al. (2013) used RNA-seq to show that the majority of the MyoD/MyoG peaks that also bind PolII and are marked by H3K4me1 and H3K27ac are transcribed in both senses in myotubes. siRNA knockdown of MyoD but not of MyoG reduced the level of these eRNAs. Among the enhancers that are transcribed in C2 myotubes, and in FACS-sorted satellite cells, are the two well-characterized *MyoD* elements, the CE and the DRR. siRNAs targeted to the CE eRNAs but not those to the DRR eRNAs caused a significant reduction in MyoD mRNA and protein indicating that transcription from the CE is required for efficient *MyoD* expression, although one cannot tell whether the eRNA acts in *cis* or in *trans*. This is an interesting demonstration that at least some

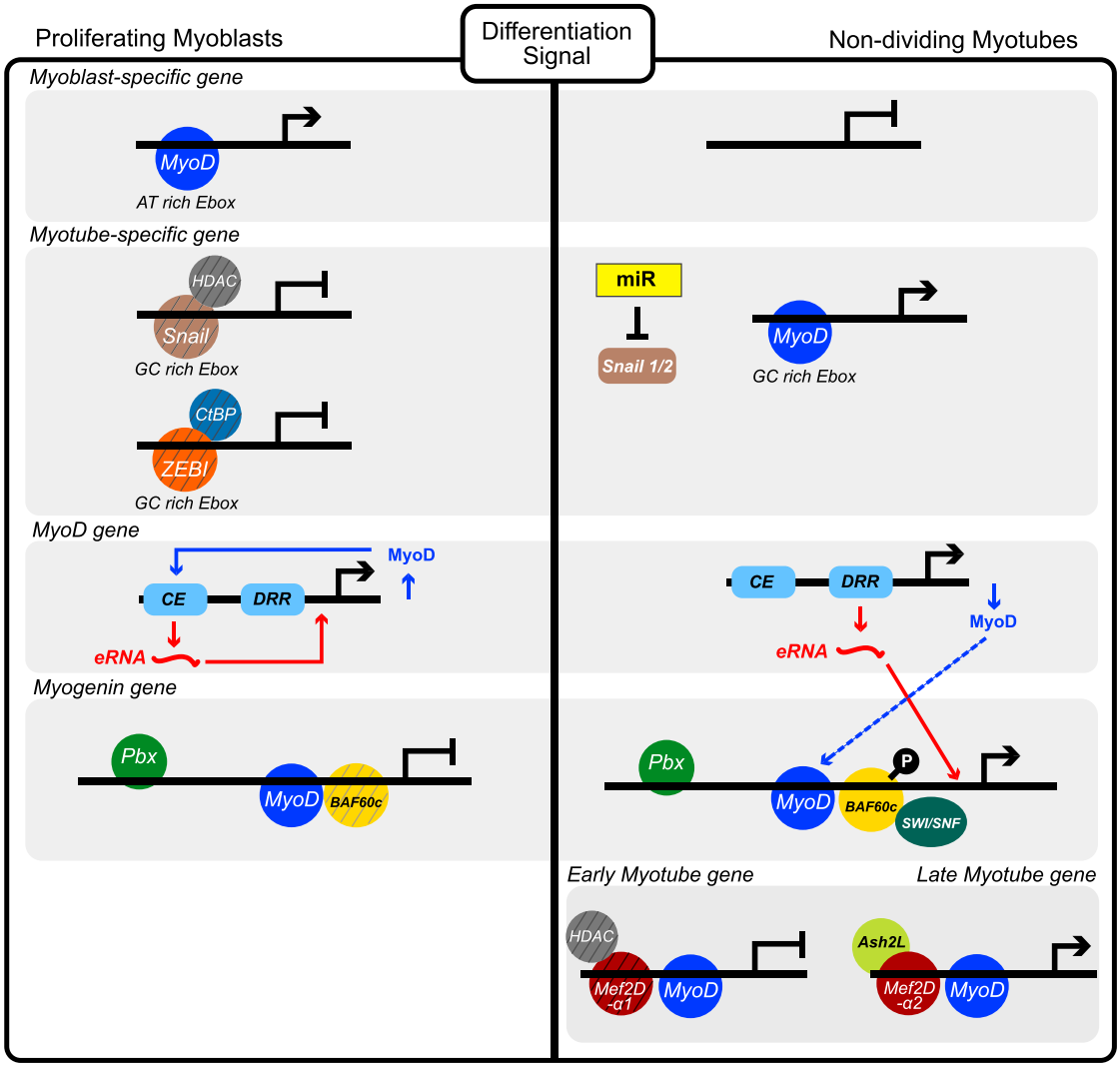

Figure 5. Schematics of Different Molecular Models for the Transcriptional Regulation of Muscle Genes in Myoblasts and Myotubes
These are based on results discussed in the review, are not comprehensive, and are not mutually exclusive. Indeed, all may operate simultaneously. Circles represent proteins binding to DNA regulatory elements; open circles are activators and hatched circles are repressors. Abbreviation: P, phosphorylated.

eRNAs are functional but the striking result comes from analogous experiments targeting the DRR eRNAs. Knocking them down prevents the activation of the MyoD target genes *MyoG* and *myosin heavy chain*. Conversely, when retroviral transduction was used to overexpress some but not all of the DRR eRNAs in C2 myoblasts, *MyoG* expression, and thus the rest of the myogenic program, was activated without any effect on MyoD transcript levels. Both types of eRNA affect chromatin accessibility at their target loci. The data thus say that eRNA from one of the *MyoD* enhancers acts in *trans* to activate the transcription of a gene well known to be a direct target of the MyoD protein. The eRNA will appear in the cell before MyoD protein, which will allow it to modulate the accessibility of the *MyoG* promoter so that it is receptive to the transcription factor. This is a novel mechanism (Figure 5) and it will be most interesting to know whether it operates in all muscle progenitors. However, it should be noted that it can not be essential for the basic function of MyoD because ectopic expression from heterologous regulatory elements will trigger the myogenic program and the DRR is dispensible for *MyoD* expression at embryonic and neonatal stages.

Regulation of the Onset of Differentiation

Proliferating myoblasts express MyoD, so why do they not differentiate until the appropriate culture medium is provided? One explanation had been the presence of Id or one of the other inhibitory proteins that prevent MyoD binding to DNA, although this is not readily compatible with the fact that *MyoD* is transcriptionally active in myoblasts, nor with the data that say that MyoD is bound to the same sites in myoblasts and myotubes. Nevertheless, somehow the transcription factor is prevented from activating those targets destined to be expressed in myotubes and Soleimani et al. (2012b) have provided a rather satisfying answer to this conundrum. As well as the bHLH proteins, E-boxes are also bound by a number of zinc-finger proteins

including the transcriptional repressor Snail, which recruits the histone deacetylases HDAC1 and HDAC2. Snail binds to E-boxes with a G/C-rich central dinucleotide, which are associated with genes expressed in myotubes but not to E-boxes with an A/T-rich central dinucleotide, which are associated with genes expressed in myoblasts. When differentiation is triggered, Snail must be removed in order to allow MyoD access. Among the genes regulated by MyoD are those encoding miR30a, which targets Snail1 mRNA, and miR206, which targets Snail2 mRNA. Overexpression of Snail blocks differentiation whereas siRNA knockdown of this repressor induces precocious differentiation. They therefore propose that when cells receive a differentiation signal, the MRFs activate the miRNAs which prevent further translation of Snail mRNAs. As the unstable Snail proteins turn over, MyoD gains access to the E-boxes of the myotube genes and activates the terminal differentiation program. Another zinc-finger protein, ZEB1, also binds to E-boxes with a G/C-rich central dinucleotide and represses transcription, in this case through association with the corepressor CtBP. ChIP experiments show that ZEB1 is bound to the promoters of selected terminal differentiation genes in myoblasts but not in myotubes and again knockdown of ZEB1 induces precocious differentiation (Siles et al., 2013). The relationship between Snail and ZEB1 function (Figure 5) is not clear from these data but should be revealed by genome-wide experiments that analyze both proteins.

Cooperation between MyoD and Other Transcription Factors

It was clear from experiments with single genes that the MRFs do not act in isolation but that they act in concert with other transcription factors, the best-characterized example being the proteins of the Mef2 family. The data from the ChIP-seq experiments provide a genome-wide view of such cooperative interactions, derived by examining the sequences around the sites of MyoD binding and asking, by a variety of methods, what other transcription factors could bind there. As expected, there are frequent sites for proteins like Mef2 and RUNX, which have been previously studied in the context of myogenesis, but also sites for proteins such as PPAR-γ and c-Myb, which have not attracted much attention from the field. MacQuarrie et al. (2013) have shown that several of the potential cooperating factors, e.g., Mef2c and RUNX1, are expressed at lower than expected levels in rhabdomyosarcoma tumor cells and that forcing their expression induces the cells to enter the terminal differentiation program. This observation provides strong biological support for the notion that they do act cooperatively, as originally proposed, on the basis of biochemical and reporter gene experiments, for the case of MyoD and Mef2 by Molkentin et al. (1995).

Alternative Splicing of Mef2

Genome-wide approaches can also provide a great deal of information about posttranscriptional regulation. Trapnell et al. (2010) performed a large-scale RNA-seq time course analysis of C2 differentiation that revealed that, in addition to the ubiquitously expressed isoform of Mef2D (Mef2Dα1), there is a muscle-specific isoform (Mef2Dα2) that appears relatively late in the differentiation process as the result of alternative splicing. Sebastian et al. (2013) have explored the biological significance of this observation and shown that overexpression of the muscle-specific isoform accelerates differentiation and can induce the expression of late genes in proliferating myoblasts. More strikingly, in an in vivo model, the ubiquitous isoform impairs regeneration whereas the muscle-specific one improves it. The two proteins bind to largely overlapping genomic sites but there are major differences in their interactomes. The ubiquitous Mef2Dα1 binds to corepressors, HDACs 4 and 9, whereas the muscle-specific Mef2Dα2 binds to the Ash2L coactivator complex (Figure 5). The choice of binding partner is controlled by protein kinase A phosphorylation; mutations that prevent this modification of the ubiquitous isoform transform it into an activator. Regulated alternative splicing can thus have a major effect on the biological activity of one of the best-characterized transcription factors involved in myogenesis.

MyoD and Chromatin Structure

It is clear from the work comparing MyoD and NeuroD that there must be lineage-specific epigenetic marks that restrict transcription factor access to chromatin. How these marks are set during early embryogenesis is a major question for the future. The activating transcription factor may be loaded in a way that is insensitive to repressive marks or it may itself be able to induce chromatin remodeling. In the first case the MRF might be loaded though interactions with a pioneer factor that can bind effectively to adjacent target gene regulatory elements that do not carry repressive marks or, more probably, in a fashion that is insensitive to them. Pbx has been shown to interact with a noncanonical homeodomain site upstream of *MyoG* in both muscle and nonmuscle cells and to facilitate the binding of MyoD to an E-box close to the transcription start site (see Tapscott, 2005). It is clear that the remodeling of chromatin structure is an important part of the process by which the MRFs activate the terminal differentiation program. MyoD binds directly to the BAF60c subunit of the SWI/SNF chromatin remodeling complex and both proteins are found at the promoters of untranscribed MyoD target genes in myoblasts. When differentiation is signaled, BAF60c is phosphorylated by p38, which allows them to be incorporated into the Brg1-based SWI/SNF complex with consequent chromatin remodeling and transcription (Figure 5; Forcales et al., 2012). Another possible mechanism for facilitating expression is the removal of repressive epigenetic marks. The histone chaperone Spt6 acts to remove nucleosomes in front of the elongating polII complex and to reassemble them once the polymerase has passed. It has recently been shown that Spt6 has another activity that recruits the histone demethylase KDM6A (UTX), which removes repressive H3K27me3 deposited by the Polycomb Repressive Complex 2. This activity is required for the activation of the terminal differentiation program in C2 cells (Wang et al., 2013), and zebrafish mutant for Spt6 have muscle defects (Kok et al., 2007). It is, of course, the case that these mechanisms are not mutually exclusive.

Role of the Core Transcription Machinery

Relatively little attention has been paid to the role of the core transcription machinery in the regulation of muscle-specific gene expression, but this an area that warrants further attention. The general transcription factor TFIID is comprised of TBP (the TATA-box binding protein) and a number of TAFs. Deato and Tjian (2007) have reported that TFIID is degraded during the differentiation of C2 cells and replaced by a simpler complex of TRF3 (a TBP-related factor) and TAF3. This new complex is required for MyoD to activate the *MyoG* promoter through a

direct interaction of TAF3 and MyoD/E47 heterodimers (Deato et al., 2008) and knockdown of either component thus blocks differentiation.

Conclusion

The genome-wide approaches have, for obvious logistical reasons, used cultured cells. However, as we have discussed, there are a number of distinct genetic programs which, in the embryo, lead to the skeletal muscle phenotype. It will be of great interest to ask detailed mechanistic questions about the activation of differentiation in each of the progenitor populations, but this will require ways of efficiently sorting the cells of interest and improvements in the sensitivity of the assays. Such data would allow us to link our understanding of the upstream regulatory networks in the embryo (Figure 3) with our knowledge of the biochemical mechanisms that lead to a skeletal muscle cell to provide a satisfying, integrated understanding of the molecular basis of myogenic determination and differentiation.

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