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The BAF60c-MyoD complex poises chromatin for rapid transcription

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Chromatin remodeling by the SWI/SNF complex is required to activate the transcription of myogenic-specific genes. Our work addressed the details of how SWI/SNF is recruited to myogenic regulatory regions in response to differentiation signals. Surprisingly, the muscle determination factor MyoD and the SWI/SNF subunit BAF60c form a complex on the regulatory elements of MyoD-targeted genes in myogenic precursor cells. This Brg1-devoid MyoD-BAF60c complex flags the chromatin of myogenic-differentiation genes before transcription is activated. On differentiation, BAF60c phosphorylation on a conserved threonine by p38 α kinase promotes the incorporation of MyoD-BAF60c into a Brg1-based SWI/SNF complex, which remodels the chromatin and activates transcription of MyoD-target genes. Downregulation of BAF60c expression prevents MyoD access to the chromatin and the proper loading of an active myogenic transcriptosome preventing the expression of hundreds of myogenic genes. Our data support an unprecedented two-step model by which (1) pre-assembled BAF60c-MyoD complex poises the chromatin of myogenic genes for rapid transcription; (2) chromatin-bound BAF60c "senses" the myogenic differentiation cues and recruits an active SWI/SNF complex to remodel the chromatin allowing transcriptional activation.

Keywords: MyoD, BAF60c, SWI/SNF, chromatin, remodeling, transcription, myogenesis, differentiation

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Binding of BAF60c and MyoD to the Chromatin of Silent Loci

Muscle differentiation is regulated by signaling pathways that repress and activate gene transcription in a spatio-temporal manner. MyoD, a master regulator of myogenic transcription, recognizes its target genes by binding the consensus Ebox sequences (a/c/g)CA(c/g)CTG and plays a fundamental role in activating transcription by recruiting chromatin modifying enzymes.[1-5] One of the questions that has been a topic of intense investigation in the myogenic transcriptional field is how MyoD gains access, in undifferentiated myoblasts, to the target DNA sequences in the repressive chromatin within the nucleosome prior to the activation of transcription.[6]

In order to address this issue, we sought to identify MyoD-binding partners that could play a role in facilitating MyoD access to the chromatin. Our work identified BAF60c as a MyoD binding partner. BAF60c is a subunit of a chromatin remodeling complex, called SWI/SNF, that uses the energy of ATP to dissociate DNA-histone contacts, therefore, we considered it to be an excellent candidate for helping MyoD access the chromatin of silent loci. Indeed and importantly, BAF60c co-localizes with MyoD at the promoter of an early myogenic differentiation marker gene (myogenin) prior to the activation of transcription without other SWI/SNF subunits such as Ini1 or Brg1 (**Fig. 1A**).[7] Furthermore, genome wide ChIP (ChIP on ChIP) showed that RNAi-mediated downregulation of BAF60c expression prevents the recruitment of MyoD to most of its target genes impairing their transcription. In turn, the absence of MyoD prevents BAF60c binding to the chromatin of myogenin. This evidence supports a model of reciprocal cooperation, whereby BAF60c and MyoD facilitate each other's binding to muscle specific loci.

Previous studies have shown that before differentiation, MyoD can bind the chromatin indirectly through the interaction with Pbx, a homeodomain factor that binds a MEF3 consensus site located upstream of the canonical Ebox at the myogenin promoter (**Fig. 1B**).[8] It is possible that BAF60c mediates the initial MyoD recruitment by facilitating interactions with Pbx. We performed ChIP experiments trying to elucidate whether the MyoD-BAF60c complex was bound to the Pbx site in vivo. However, the Eboxes and the consensus binding sequence for the Pbx protein are too close to tell them apart by ChIP analysis, even when we fragmented the chromatin to 150 bp. Consequently, we were able to monitor MyoD-BAF60c binding at the myogenin promoter, but not determine the specific regulatory sequence element responsible for this recruitment. To better understand the dynamics of MyoD recruitment to the chromatin, it would be very interesting to monitor MyoD binding to transfected myogenin promoter constructs containing combinations of mutated regulatory sequences (Ebox, Pbx, MEF2 and Six1). These constructs could be luciferase reporter vectors (to check for transcriptional activity) and importantly, should integrate into the genome or at least mimic the endogenous chromatin structure (such as the chromatin-forming episomal pREP4-luc vector). Downregulating the expression of BAF60c or Pbx in the transfected cells could address which are the specific sites bound by MyoD in vivo before and after differentiation, as well as the roles of Pbx and BAF60c in facilitating this binding. Furthermore, ReChip experiments could also define the reciprocal interactions between BAF60c, MyoD and Pbx at the chromatin level. Ideally, the best approach would be to mutate the endogenous consensus-binding sites by in vivo site-directed mutagenesis methods,[9] although these are still challenging techniques.

Chromatin-Bound BAF60c in Silent Loci is Devoid of Catalytic SWI/SNF Subunits

We were surprised to find a subunit of the SWI/SNF complex binding the chromatin of early target genes (e.g., myogenin) in

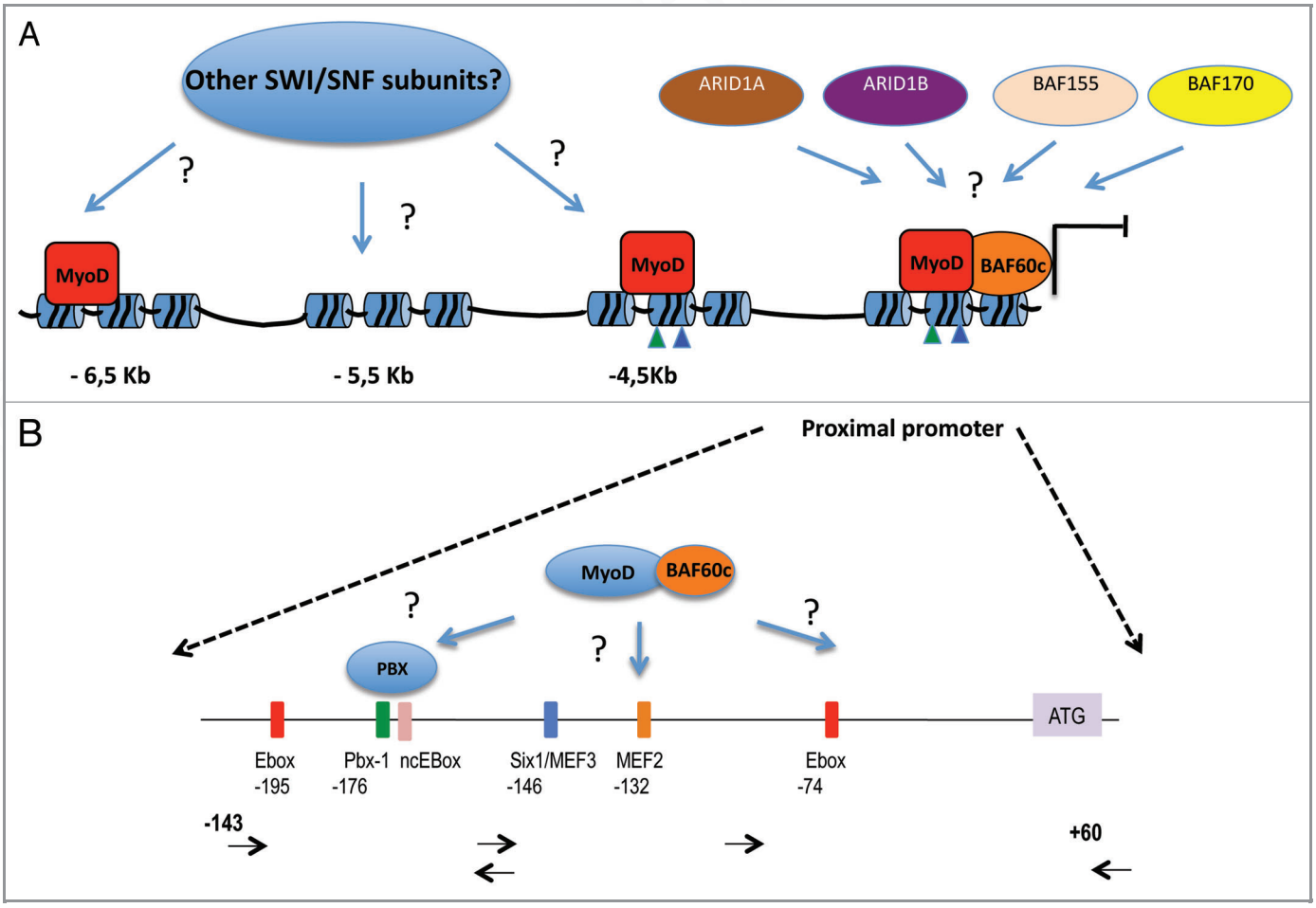

Figure 1. Illustration of the myogenin promoter. (A) In myoblasts, before differentiation signals arrive, the chromatin of myogenin, is bound by BAF60c-MyoD complex at the promoter regions in the absence of Ini1 or the catalytic subunits Brg1 and Brm. The presence of other SWI/SNF subunits such as BAF155, BAF170 or ARIDs and their possible contribution to repression is unknown. MyoD also binds to myogenin enhancer regions (-6.5 Kb and -4.5 Kb) however the binding of BAF60c or other SWI/SNF subunits to the enhancer regions has not been demonstrated. (B) Several regulatory DNA elements are shown in the myogenin proximal promoter. Arrows indicate the primers used for ChIP and Rechip assays that were used in an attempt to tell apart binding regulatory sequences for the MyoD-BAF60c complex.

the absence of the SWI/SNF catalytic subunit Brg1, which is required for the activation of these genes.[1,2] Interestingly, individual SWI/SNF subunits have been shown to bind to the chromatin of silent and active genomic loci,[10] although the function of these chromatin-bound-SWI/SNF subunits has not been deeply addressed. Our work revealed a previously unidentified function of the chromatin-bound SWI/SNF subunit, BAF60c, in the activation of muscle gene transcription. We showed that BAF60c helps MyoD to gain access to the chromatin. Future studies will establish whether this is due to the induction of changes in the 3D conformation structure of the proteins when they interact, conferring on them a higher affinity for the target chromatin. Alternatively, MyoD-BAF60c-induced regional chromatin changes could facilitate DNA-protein interactions and stabilize MyoD binding to target genes.

Rather than a direct repressor role for the MyoD-BAF60c interaction at the chromatin of silent myogenic genes, we suggest that the concomitant presence of BAF60c and MyoD poises these regions for a rapid transcriptional activation when the differentiation signals arrive. Therefore, we propose that the MyoD-BAF60c complex provides an epigenetic memory of poised genes during myoblast proliferation. Supporting this idea, down-regulation of BAF60c by RNAi does not activate myogenin transcription at any time in undifferentiated, proliferating myoblasts. Indeed, the absence of BAF60c prevents MyoD binding and histone H3 acetylation, while it does not decrease the presence of typical repressor histone modifications such as H3K9me3 and H3K27me3 at the myogenin locus prior to the activation of transcription. In agreement with the idea that MyoD-BAF60c could induce a poised or intermediate chromatin state, recent data by ChIP-seq[11] shows that MyoD binding to the chromatin is associated with histone H4 acetylation even at silent loci, which suggests that MyoD-BAF60c chromatin binding co-occurs with a more relaxed or open chromatin, an intermediate/ready-to-go state that may be easier and faster to remodel as soon as the differentiation signals arrive.

In support of a potential repressor role of specific SWI/SNF combinations, it has been shown that SWI/SNF complexes containing the catalytic subunit Brm prevent transcriptional activation during osteoblast differentiation by binding the HDAC1 co-repressor.[12] Brm and BAF57 have been shown to form a co-repressor complex with a methyl-CpG binding protein (MeCP2) involved in gene silencing.[13] Additionally, recent work showed that BAF57 inhibits MyoD-dependent activation of myogenin transcription through interaction with the Teashirt-3 protein;[14] however, the interaction of BAF57 with the myogenin promoter was not shown and therefore more research is needed to understand if this is a direct or indirect repressor mechanism. In agreement with these findings, we could not detect Brm associated with the MyoD-BAF60c complex at the myogenin promoter prior to activation of transcription, therefore, further supporting a poised-activator role rather than a repressor role for the MyoD-BAF60c complex. The absence of several core SWI/SNF subunits such as Brg1, Brm and Ini1/BAF47 in the MyoD-BAF60c complex detected on the chromatin of muscle genes in myoblasts is particularly puzzling, and indicates the importance of heterogeneity and dynamic exchange of SWI/SNF composition.[15] Further analyses will determine the precise composition and function of SWI/SNF complexes sequentially assembled at MyoD target genes, whether this heterogeneity might impart repressor/activator activities, and the relative role of BAF60c in SWI/SNF complex assembly.

BAF60c-Mediated Recruitment of an Active SWI/SNF Complex by Differentiation-Activated p38 Kinase

Several studies show that transcriptional factors and cofactors interact with SWI/SNF subunits, enabling the SWI/SNF complex to recognize specific loci in the genome.[16] While certain interactions might be predictive of specific signaling pathways controlling SWI/SNF function, the identity of the enzymes that promote specific posttranslational modifications at the SWI/SNF subunits remain largely unknown. Additionally, how these modifications modulate the activity and genomic redistribution of SWI/SNF is poorly understood.[17,18]

It is well known that p38 MAPK plays a fundamental role in the transition of myoblast to differentiated myocytes, as p38 activity is essential for myogenesis.[19-21] We had previously reported that SWI/SNF recruitment to myogenic regulatory regions was dependent on p38 activity, and that p38 kinases α and β could phoshorylate a SWI/SNF subunit with a predicted size of one of the three BAF60 proteins in vitro.[2] However, the identity of the specific BAF60 variant targeted by p38 α/β-mediated phosphorylation, and the in vivo role of this event remained unanswered. To address these questions, we analyzed the BAF60 family of SWI/SNF subunits and only BAF60c contained a suitable phospho-acceptor site for p38 α/β kinases. Notably, this phosphorylation target motif appears conserved in human, rat and mouse BAF60c sequences and was not present in BAF60a or BAF60b. This site was a Threonine Proline-directed residue in position 229. Mutation of this site to an Alanine impaired the incorporation of the phosphorylation in an in vitro kinase assay, confirming that it was the acceptor site for p38-direct phoshorylation. Furthermore, in vivo experiments confirmed this Threonine as the "sensor" of p38 activity since overexpression of a wild-type BAF60c induced an earlier and strong myogenic differentiation (formation of large multinucleated myotubes) while the mutant induced a weaker and delayed response. At the chromatin level, the BAF60c phosphorylation-deficient mutant facilitates MyoD binding in the same manner as the BAF60c wild-type does, however, the mutant BAF60c is unable to recruit an active SWI/SNF complex to the myogenin promoter and thus myogenin transcription is impaired. In agreement with these results, our previous work[2] showed that p38 α/β kinase inhibition by soluble compounds (SB203580) prevented BAF60c phosphorylation and consequent Brg1 recruitment to the myogenic loci. In this scenario, histone acetylation and the recruitment of factors such as MyoD and acetyltransferases (p300, PCAF) were unaltered, revealing the specificity of

p38-mediated phosphorylation in mediating recruitment of Brg1-based SWI/SNF complex to MyoD-target genes. All these data underscore BAF60c-phosphorylation as a specific event that directs SWI/SNF recruitment to the chromatin subjected to active remodeling during cellular differentiation. Other SWI/SNF subunits may be modified post-translationally by

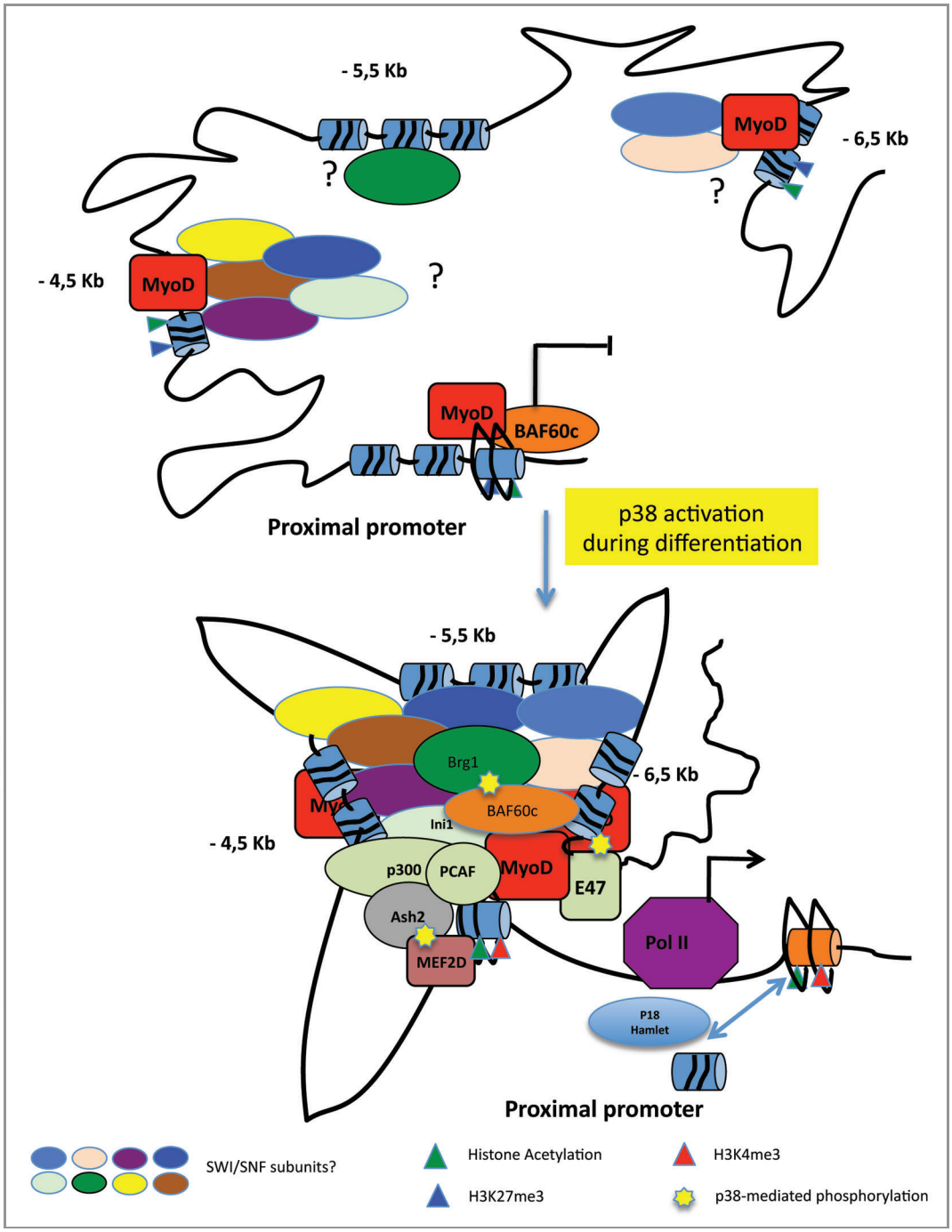

Figure 2. Diagram of an enhancer-promoter looping hypothetical model during transcriptional activation of myogenin. Before differentiation, MyoD-BAF60c flags the promoter of myogenin and could induce a locally relaxed chromatin, a surface easier recognizable for the SWI/SNF complex when differentiation signals arrive (p38 kinase activation). Since we have shown that BAF60c is present in the promoter without other subunits (Brg1, Brm or Ini1) it would not be surprising to find that these and other SWI/SNF subunits are pre-assembled in enhancer regions before differentiation signals arrive (A). Differentiation-activated p38 kinase phosphorylates BAF60c and this event is crucial for recruiting an active SWI/SNF complex to the promoter. It is possible that enhancer and promoter regions form loops allowing the binding of a complete SWI/SNF complex that will remodel the chromatin (B). In addition, p38-dependent (recruitment of MEF2D, ASH2 and MyoD-E12 heterodimerization) and p38-independent events (recruitment of p300/PCAF) occur which induce histone modifications and establish the myogenic transcriptosome that will activate transcription.

other enzymes. Future proteomic studies will help to reveal the nature of these modifications and their mediators.

In our work, we have not only described that SWI/SNF is recruited to myogenic loci in response to the differentiation-activated p38 pathway, but importantly, we have identified the SWI/SNF subunit being directly phosphorylated by p38 kinase (BAF60c), and determined the functional impact of this phosphorylation on myogenic differentiation. While BAF60c phosphorylation is crucial for initiating the chromatin remodeling that leads to transcriptional activation, there are still some questions regarding the nature of this recruitment. For instance, genome-wide studies are needed to determine the possible interactions of SWI/SNF subunits with enhancer regions and their possible role in looping and long-distance interactions with promoters, as shown with the CIITA promoter where various combinations of BAF155, BAF170, Ini1 and Brg1 have been detected at different enhancer elements and therefore could mediate these looping interactions.[10,22] Recent work by ChIP-seq analysis has revealed that MyoD binds Eboxes present in enhancer regions of the myogenin gene located at -6.5 Kb and -4.5 Kb.[11] Furthermore, epigenomic data shows that RNApol II is present in some of these enhancer regions.[23] Therefore, it would not be surprising to find other SWI/SNF subunits bound in distal areas of the myogenin gene that are recruited to the promoter region following p38-mediated phosphorylation of BAF60c. Integrating genome-wide data from ChIP, nucleosome positioning and chromosome capture conformation techniques at several time points of differentiation could establish if there are pre-assembled SWI/SNF subunits at different myogenin regulatory regions (or other genomic loci) that come together to form a complete active SWI/SNF complex in response to p38 signaling (**Fig. 2**). This model could explain the rapid recruitment of an active SWI/SNF complex following p38 activation and the need to place subunits of the same complex distributed in genomic regulatory regions that come together on activation signals.

Conclusions and Future Perspectives

Our work helped to move the understanding of the mechanisms by which MyoD activates transcription from muscle loci forward. First, we have shown that a subunit of the SWI/SNF chromatin remodeling complex, BAF60c, binds the promoter of an early marker of skeletal muscle differentiation, myogenin, in the absence of catalytic SWI/SNF subunits and before transcription is activated. At this time, BAF60c and MyoD bind the chromatin of myogenic genes together in progenitor cells and they mutually facilitate binding. This pioneering event poises the chromatin for rapid transcriptional activation. Second, a p38-dependent phosphorylation of BAF60c is the key event that senses differentiation at the chromatin level, recruiting an active SWI/SNF complex that will remodel the nucleosome, and allow transcriptional initiation. Our proposed 2-step model for remodeling myogenic loci is in accordance with a stochastic assembly model proposing intermediate interactions for large multisubunit complexes that have also been described for spliceosomes, DNA repair complexes and RNA polymerases with associated transcription factors.[24,25]

Knowledge of transcriptional regulation has advanced dramatically in the last decade; the identification of the histone code and the integration of genome-wide and proteomic techniques have been crucial for revealing new revolutionary mechanisms of transcriptional regulation. Nevertheless, many aspects of the transcriptional process remain unexplained. In the myogenic transcription some exciting questions remain to be addressed. How is chromatin architecture integrating regulatory factor binding and tethering to the chromatin? If loops are bringing together enhancer and promoter regions, who are the mediators and which are the genes or genomic domains being regulated in this fashion? What are the specific kinetics of regulatory factor binding that modulate transcriptional regulation? Does chromosomal 3D structure vary between proliferating and differentiating myoblasts?

Regarding BAF60c functions, it would be important to identify new BAF60c chromatin target sites; to monitor if BAF60c binds MyoD-independent loci; whether BAF60c chromatin binding is accompanied by other SWI/SNF subunits and its significance/impact for chromatin structure and regulation.

Furthermore, it is crucial to understand the role that different combinations of preassembled SWI/SNF components may play in regulating chromatin architecture and remodeling.

Immunoprecipitation assays followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) could be used to identify differentiation-stage specific BAF60c-complexes. This approach could also identify post-translational modifications in BAF60c and in other SWI/SNF subunits that can play an important role in transcriptional activation/repression. In addition, the identification of BAF60c-interacting enzymes that promote these modifications could also reveal new regulatory pathways that may be involved in myogenic transcription.

Finally, it would be very interesting to explore in detail how the nucleosomes are remodeled along the myogenin promoter and how nucleosome location genome-wide differs in precursor vs. differentiated cells. Do BAF60c-differential complexes have an impact on DNA accessibility and how?

In summary, continued efforts to investigate these molecular mechanisms are needed and will be crucial for a better understanding of transcriptional regulation.

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