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The RNA Helicases p68/p72 and the Noncoding RNA SRA Are Coregulators of MyoD and Skeletal Muscle Differentiation

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Summary

MyoD regulates skeletal myogenesis. Since proteins associated with MyoD exert regulatory functions, their identification is expected to contribute important insights into the mechanisms governing gene expression in skeletal muscle. We have found that the RNA helicases p68/p72 are MyoD-associated proteins and that the noncoding RNA SRA also immunoprecipitates with MyoD. In vitro and in vivo experiments indicated that both p68/p72 and SRA are coactivators of MyoD. RNA interference toward either p68/p72 or SRA prevented proper activation of muscle gene expression and cell differentiation. Unexpectedly, reducing the levels of p68/p72 proteins impaired recruitment of the TATA binding protein TBP; RNA polymerase II; and the catalytic subunit of the ATPase SWI/SNF complex, Brg-1, and hindered chromatin remodeling. These findings reveal that p68/p72 play a critical role in promoting the assembly of proteins required for the formation of the transcription initiation complex and chromatin remodeling.

Introduction

The specification of different cell lineages starting from totipotent stem cells lies at the core of development and organogenesis. During this process, the genome of a cell is modified to ensure that stable, if not irreversible, distinctions are made between genes not to be expressed from genes whose expression is or will be required. At the molecular level, these distinctions are brought about by specific modifications of the chromatin. Indeed, the developmental activation of genes expressed in highly differentiated cell types is characterized by a defined chromatin conformation (Weintraub and Groudine, 1976) that depends on the interaction of *trans* regulatory proteins (transcriptional regulators) with *cis* DNA sequences (regulatory regions). DNA binding of transcriptional regulators is a prerequisite, but it is insufficient to initiate transcription. To effectively promote gene activation, transcriptional regulators recruit proteins that, while per se incapable of recognizing specific DNA modules, can modify the structure and function of the chromatin and of the transcriptional regulators themselves. Thus, gene expression is regulated by both DNA binding of transcriptional regulators and by the composition of the associated protein complexes.

MyoD and the related myogenic bHLH proteins specify and direct differentiation of the skeletal muscle lineage in mice (Sabourin and Rudnicki, 2000; Buckingham et al., 2003) by interacting with a specific DNA module, the E-box, located in the regulatory regions of genes expressed in skeletal muscle (Lassar et al., 1989, 1991). In this process, the MEF2 proteins cooperate with the myogenic bHLH proteins (Molkentin et al., 1995). The regulation of MyoD activity is complex and only partially understood (Tapscott, 2005; Sartorelli and Caretti, 2005). While binding of MyoD to its DNA targets is generally followed by gene activation, some target genes are bound and not activated (Ohkawa et al., 2006) or are activated with different kinetics (Bergstrom et al., 2002; Blais et al., 2005; Ishibashi et al., 2005; Cao et al., 2006). Moreover, MyoD can repress gene expression (Bergstrom et al., 2002). Association of MyoD and MEF2 with histone modifiers and chromatin remodeling complexes is believed to regulate their transcriptional activity. Interaction of class I and class III histone deacetylases (HDACs) with MyoD (Mal et al., 2001; Puri et al., 2001; Fulco et al., 2003) and of class II and class III HDACs with MEF2C (Zhang et al., 2002; Zhao et al., 2005) may prevent premature gene activation in undifferentiated myoblasts. Moreover, methylation of specific histone lysine residues at muscle loci (Zhang et al., 2002; Caretti et al., 2004; Mal and Harter, 2003) likely reinforces the inhibitory effects exerted by the HDACs. At the onset of differentiation, the composition of the protein complexes interacting with the myogenic bHLH and MEF2 proteins is modified to accommodate recruitment of the chromatin remodeling SWI/SNF, p300, and PCAF histone acetyltransferases

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to promote gene activation (McKinsey et al., 2002; Forcales and Puri, 2005). Because of the regulatory functions exerted by the proteins associated with MyoD, the identification and characterization of the individual components of these protein complexes are expected to contribute important insights into the mechanisms governing gene expression in skeletal muscle.

In this study, we report that the RNA helicases p68/p72 are MyoD-interacting proteins and that the noncoding RNA steroid receptor RNA activator (SRA) can be coimmunoprecipitated with MyoD. p68/p72 and SRA coactivate MyoD-dependent transcription and are required for skeletal muscle cells to properly differentiate. Unexpectedly, we found that p68/p72 are mechanistically linked to the productive recruitment of proteins required for the formation of the transcription initiation complex, chromatin remodeling, and transcriptional activation of muscle loci.

Results

MyoD Interacts with p68 and p72, Two Members of the DEAD-Box Family of RNA Helicases

To facilitate the purification and identification of proteins that associate with MyoD, HeLa cells were transduced with the pOZ retrovirus expressing a bicistronic mRNA encoding Flag and HA epitope-tagged MyoD linked to the ILR2 α subunit surface marker via an internal ribosomal entry site. The transduced cells were purified by repeated cycles of affinity cell sorting on magnetic beads coated with an IL2α receptor antibody and uniform positive reactivity of the cell population to both MyoD and IL2α confirmed by immunofluorescence microscopy and FACS analysis. Because we employed a low viral titer, the level of MyoD protein expressed in HeLa cells was comparable to that detected in C2C12 skeletal muscle cells (data not shown). Nuclear extracts were prepared and purified by affinity chromatography on anti-Flag antibody-conjugated agarose. The bound material was first eluted with the epitope Flag peptide under native conditions and was subsequently repurified on a second anti-HA antibody-conjugated agarose. Polypetides were eluted from the resin by denaturing with SDS and heat. As a control, the same purification scheme was performed with nuclear extracts obtained from nontransduced HeLa cells.

Several polypeptides copurified specifically with the epitope-tagged MyoD (e-MyoD) (Figure 1A, compare lanes 3 and 5), and their identity was determined by mass spectrometry. Numerous MS/MS spectra were assigned to peptides belonging to proteins known to interact with MyoD, such as E proteins (Murre et al., 1989), class I HDACs (Mal et al., 2001; Puri et al., 2001), and the corepressors NcoR/SMRT (Bailey et al., 1999) (data not shown and Figure S1A; see the Supplemental Data available with this article online). Another set of MS/MS spectra corresponded to peptides of proteins not previously reported to interact with MyoD. Among these, we focused on the set of peptides whose sequences are shown in Figure 1B and that were assigned to the two RNA helicases, p68 and p72, with high sequence homology (Figure 1C). We confirmed that the peptides identified by mass spectrometry corresponded to the p68 protein by immunoblotting (Figure 1D). The strength of the interaction of p68 with MyoD was comparable to that of HDAC3 and NcoR, two other proteins identified by mass spectrometry as MyoD-associated factors (Figure S1A). Recognizing the physiological limitations of the cellular system employed to isolate MyoD-associated factors, we evaluated whether p68/p72 may interact with MyoD in extracts of skeletal muscle cells. Endogenous MyoD immunoprecipitated from nuclear extracts of differentiated C2C12 muscle cells interacted with endogenous p68 (Figure 1E) and p72 (data not shown). The results reported in this paragraph suggest that p68/p72 are bona fide MyoD-associated factors.

The p68 and p72 Proteins and the Noncoding RNA Steroid Receptor Activator SRA Are Transcriptional Coactivators of MyoD

The mass spectrometry protocol employed to identify MyoD-associated factors is not suited to reveal the presence of nucleic acids. p68/p72 are RNA binding proteins that interact with a regulatory noncoding RNA, the SRA (Watanabe et al., 2001). Before initiating an investigation into the role of p68/p72 in myogenesis, we wished to determine whether SRA might be associated with MyoD and/or p68. Expression vectors for either Flag-MyoD or Flag-p68 were transfected in 293 T cells, and the cell extracts were immunoprecipitated with an anti-Flag antibody-conjugated agarose or control immunoglobulin. The immunoprecipitated material was treated with DNaseI to eliminate potentially present genomic DNA and was subsequently subjected to a reverse transcription reaction. The cDNA was PCR amplified with primers specific for either SRA or, as a negative control, tubulin. The results shown in Figure 2A and Figure S1D indicate that p68 and MyoD coimmunoprecipitated RNAs corresponding to SRA sequences. Moreover, in extracts derived from HeLa-Flag-MyoD cells, but not control HeLa cells, SRA was immunoprecipitated by anti-Flag antibodies (Figure S1B). Similar experiments conducted with differentiated myotubes revealed that SRA is associated with endogenous p68 or MyoD in skeletal muscle cells (Figure 2B). Reactions in which the reverse transcription step was omitted did not give signals above background (data not shown). The association of MyoD and p68 is unlikely to be mediated by SRA since treatment with RNase did not prevent the interaction of the two proteins in nuclear extracts of differentiating C2C12 cells (Figure S1E). Real-time PCR (RT-PCR) performed on RNase-treated nuclear extracts failed to amplify transcripts corresponding to SRA, indicating that RNase treatment was successful (data not shown). The observation that MyoD, p68/p72, and SRA have the potential to form a protein-RNA complex prompted us to test for their functional relevance to muscle gene transcription. To this end, the muscle-specific creatine kinase enhancer linked to the luciferase gene (MCK-luc) construct was transfected in NIH3T3 murine fibroblasts. Transfection of either individual or pairwise combinations of p68, p72, and SRA expression vectors had no effect on MCK-luc in the absence of MyoD (Figure 2C). Conversely, MyoD-dependent transactivation of MCK-luc was increased, in a dose-dependent manner, by coexpression of either p68 or p72. SRA, either alone or, more effectively, in combination with p68 or p72, also augmented MyoD-dependent

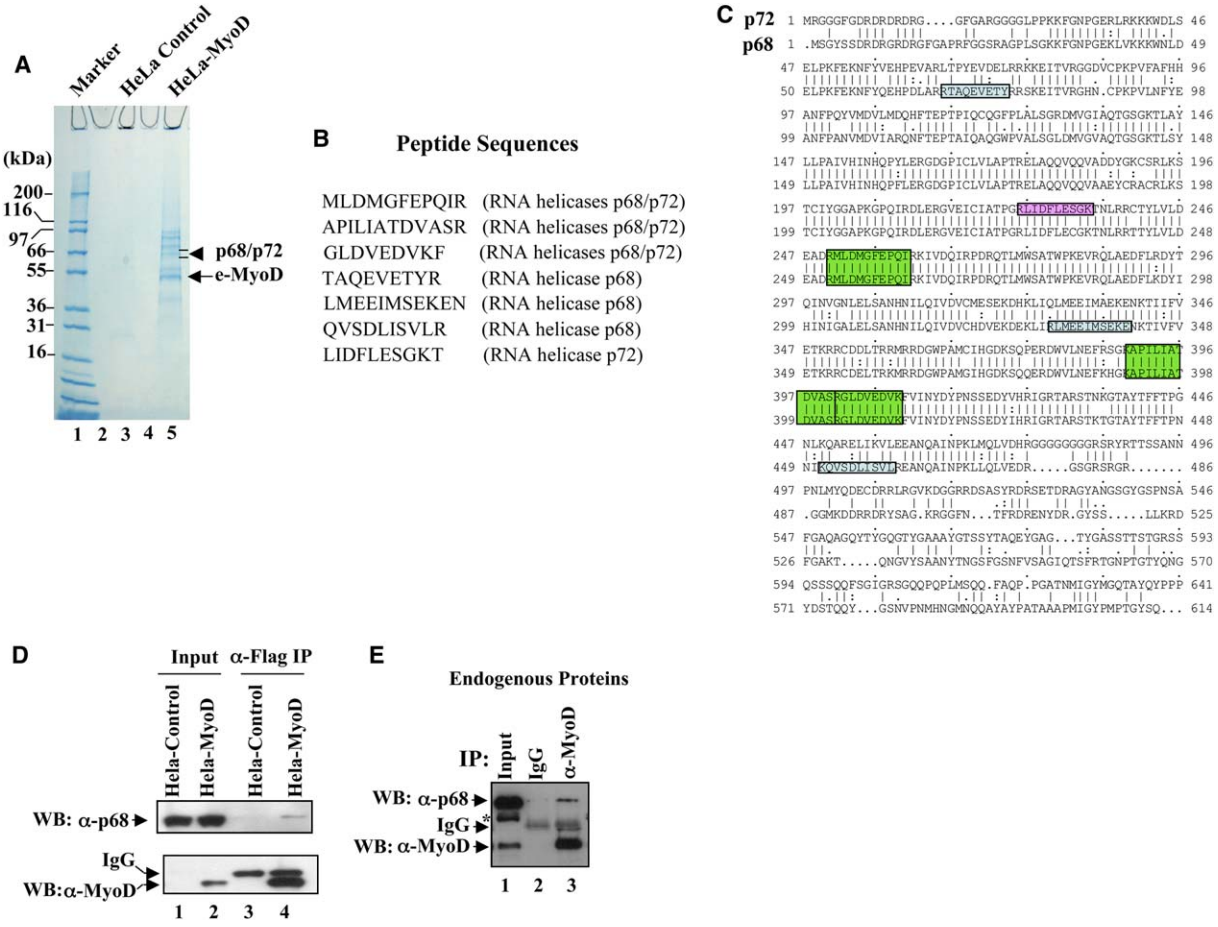

Figure 1. Purification and Identification of p68/p72 as MyoD-Associated Proteins

(A) Nuclear extracts were prepared from either mock-transduced control (lane 3) or MyoD-expressing HeLa cells (lane 5) and purified by affinity chromatography as described by Nakatani and Ogryzko (2003). Proteins were resolved by SDS-PAGE, and the gel was stained with Simply Blue (Invitrogen). The arrowhead points at bands later identified as p68/p72, and the arrow points to epitope-tagged MyoD (e-MyoD).

(B) Peptide sequences identified by mass spectrometry and corresponding to human RNA helicase p68/p72 proteins.

(C) Alignment of human p68/p72 proteins. The light-blue boxes identify p68-specific peptide sequences, the pink box indicates a p72-specific peptide, and the green boxes encompass common p68/p72 peptide sequences.

(D) Nuclear extracts from either mock-infected control (lane 3) or MyoD-expressing HeLa cells (lane 4) were immunopurified on anti-Flag antibody-conjugated agarose, and the materials were resolved by SDS-PAGE and immunoblotted with p68 and MyoD antibodies. A total of 20% of the total protein inputs for control (lane 1) and MyoD-expressing (lane 2) HeLa extracts.

(E) Nuclear extracts of C2C12-differentiated muscle cells were immunopurified with either control IgG (lane 2) or MyoD antibody (lane 3), and the materials were resolved by SDS-PAGE and immunoblotted with a p68 antibody. The asterisk indicates a band often observed to react with p68 antibody and, most likely, generated by partial proteolysis of the p68 protein. Lane 1 shows 10% of the protein input employed in lanes 2 and 3.

transactivation. NIH3T3 cells express p68 (Figure S2A) to assist SRA-mediated coactivation of MyoD in the absence of transfected p68 (Figure 2C). The strongest activation of MCK-luc was observed when p68, p72, and SRA were simultaneously cotransfected with MyoD. An expression vector bearing a substitution of a single amino acid of p68 (K144R) located in the ATP binding domain, which is also conserved in the ATP binding domain of p72 (Lamm et al., 1996) and inactivates p68's RNA helicase activity (Endoh et al., 1999), continued to coactivate MyoD-dependent transcription, either in the absence or presence of transfected SRA (Figure 2D), indicating that the helicase activity of p68 is dispensable to regulate MCK-luc transcription. SRA expressed in antisense (3′-5′) orientation failed to coactivate MyoD (Figure S2B). As reported for the bHLH E47 protein (Lanz et al., 1999), SRA was also incapable of coactivating transcription mediated by the bHLH E2-5 (ITF-1), a protein related to, but distinct from, E47 (Figure S2C). Recently, *Drosophila* p68 has been reported to regulate transcriptional deactivation by promoting RNA release from chromatin (Buszczak and Spradling, 2006). These findings raise the possibility that the effects exerted by p68 on MyoD-dependent transcription may be indirect, i.e., p68 may deactivate transcription of a MyoD inhibitor and, consequently, favor MyoD activity. To rule out both this and other possible indirect effects of p68/p72 and SRA on MyoD-dependent transcription, we evaluated their ability to activate muscle gene transcription in a MyoD-dependent in vitro transcription system based on a chromatin-reconstituted template. A DNA template encompassing four copies of a MyoD binding site derived from the MCK enhancer linked to the MCK promoter (4R-MCK) was incorporated into nucleosomal

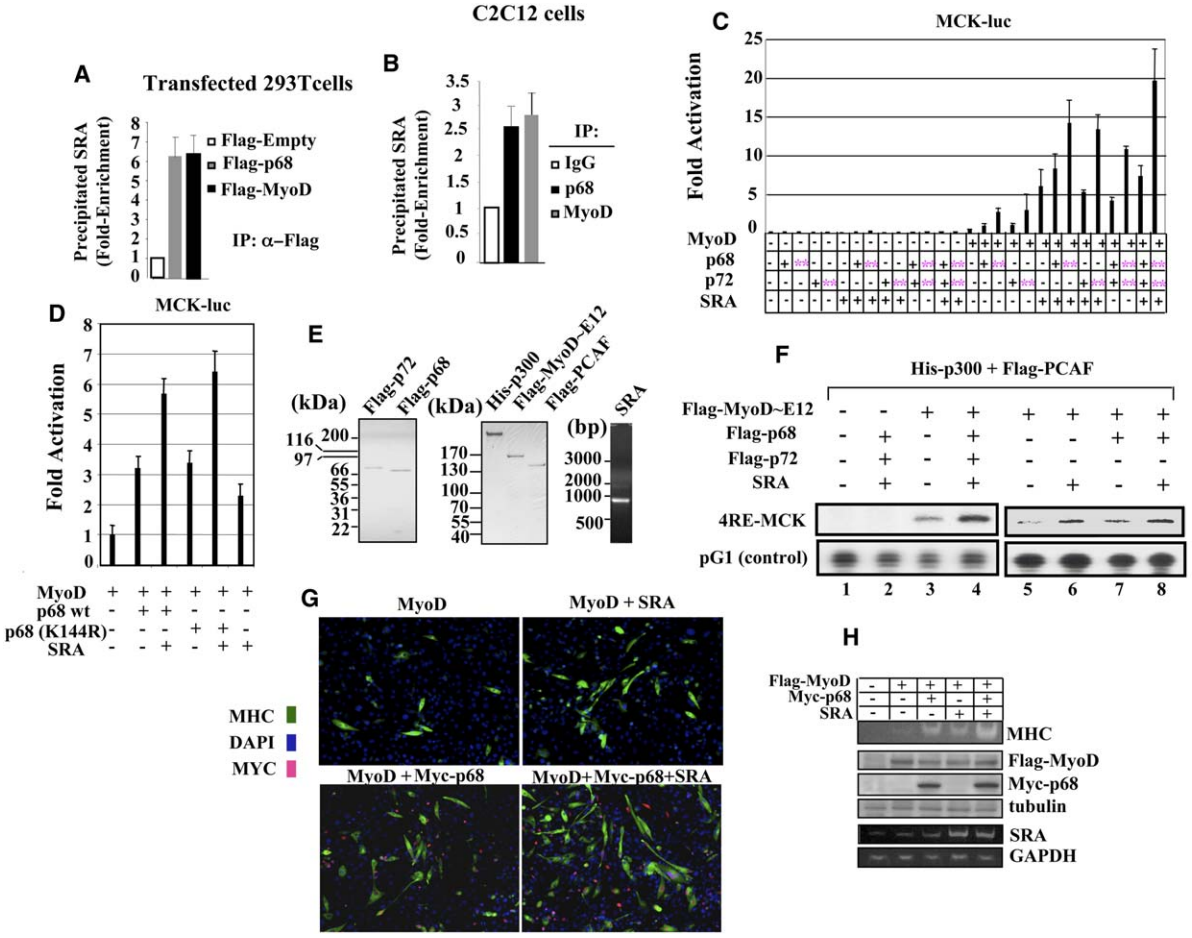

Figure 2. p68/p72 and the Noncoding RNA Steroid Receptor Activator, SRA, Coactivate MyoD-Dependent Transcription

(A) 293 T cells were transfected with the indicated Flag-tagged expression vectors, and cell extracts were prepared and immunopurified on anti-Flag antibody-conjugated agarose. The immunopurified materials were first treated with DNaseI and then subjected to RT-PCR with specific primers for SRA. As control, the same materials were also amplified with specific primers for tubulin transcripts. The values obtained with RT-PCR analysis for SRA amplifications were normalized to the values obtained for tubulin amplifications. Error bars represent standard deviations.

(B) Experiments were conducted as described in (A), except that cell extracts from C2C12 skeletal muscle cells immunopurified with either p68 or MyoD antibodies were employed. Error bars represent standard deviations.

(C) NIH3T3 fibroblasts were transfected with the MCK-luc reporter and expression vectors for MyoD, p68, p72, and SRA in different combinations. Two different concentrations (50 ng, +; 250 ng, ++) of p68/p72 plasmids were employed, while concentrations of the MyoD (20 ng) and SRA (50 ng) plasmids were kept constant. A luciferase assay was conducted on cell extracts lysed 48 hr after transfection. A total of 50 ng of a CMV-lacZ-expressing vector was cotransfected, and a β–gal assay was performed to correct for transfection efficiency. Experiments were done with triplicate samples and were repeated 4–5 times. Error bars represent standard deviations.

(D) Expression vectors for MyoD, wild-type (wt) p68, and helicase-deficient p68 (p68 K144R) were transfected in NIH3T3 cells in the presence of the MCK-luc reporter, and luciferase activity was determined as described in (C). Error bars represent standard deviations.

(E) Recombinant proteins used for the in vitro studies, except for His-p300, which was purified by using Ni^{2+}-NTA resin, were expressed in baculovirus-infected Sf-9 cells and purified by anti-Flag affinity chromatography. The homogeneity of the purified proteins was confirmed by Coomassie blue staining after SDS-PAGE. SRA was transcribed as reported in Experimental Procedures, and an aliquot was analyzed on a 1% agarose gel after ethidium bromide staining.

(F) Flag-MyoD ~ E12 transcription from the nucleosomal 4R-MCK template was examined in the absence or presence of Flag-p68, Flag-p72, and SRA. Inclusion of Flag-p68, Flag-p72, and SRA increased MyoD ~ E12-mediated transcription by ~5-fold (compare lanes 3 and 4). SRA alone increased Flag-MyoD ~ E12 transcription by ~4-fold (compare lanes 5 and 6). Flag-p68 increased MyoD ~ E12 transcription by ~2-fold (compare lanes 5 and 7). His-p300, Flag-PCAF, and acetyl-CoA were present in every reaction. Transcription from the pG1 template served as an internal control. Quantification of the in vitro transcription reactions was performed by scanning the gel autoradiogram with the NIH ImageJ software.

(G) C3H10T1/2 mouse embryonic fibroblasts were transfected with different combinations of MyoD, Myc-p68, and SRA expression vectors, and their myogenic conversion was scored by immunostaining with myosin heavy chain (MHC, green) antibodies after culturing them for 72 hr in differentiation medium. Reactivity with the myc-epitope of the Myc-p68 protein is indicated in red, and DAPI (blue) marks the cell nuclei.

(H) The levels of MHC transcripts were investigated by RT-PCR, expression of Flag-MyoD and Myc-p68 in transfected C3H10T1/2 cells was evaluated by immunoblotting, and expression of SRA in transfected C3H10T1/2 cells was evaluated by RT-PCR. Tubulin protein and GAPDH RNA served as loading controls.

arrays, and baculovirus-produced and -purified MyoD fused to its physiological protein partner E12 (MyoD∼E12), p68/p72 proteins, and in vitro-transcribed SRA (Figure 2E) were assayed in a transcription reaction. Formation of the nucleosomal arrays was confirmed by micrococcal nuclease digestion (data not shown). The acetyltransferase p300 and PCAF proteins were included in the transcription reaction, as they have been shown to be required for MyoD∼E12-dependent transactivation (Dilworth et al., 2004). The pG1 template, containing the β-globin gene promoter, was employed as an internal control. Positioning of nucleosomes on the 4R-MCK template established a transcriptionally repressed state on the promoter region since no transcription was observed from the nuclear extract in the absence of MyoD∼E12 protein (Figure 2F, lanes 1 and 2). While the addition of MyoD∼E12 (in the presence of a nuclear extract) was sufficient to overcome the repressive effects on transcription (lane 3), the addition of Flag-p68, Flag-p72, and SRA resulted in a further 5-fold increase in transactivation (compare lanes 3 and 4). SRA on its own was able to increase MyoD∼E12-dependent transactivation ∼4-fold (lane 6), while p68 demonstrated a weaker, but very reproducible, increase in transactivation in the presence of MyoD∼E12, alone or in combination with SRA (lanes 7 and 8). The limited increase in transactivation observed in the presence of p68 may be due to the large amount of the RNA helicases (both p68 and p72) present in the HeLa nuclear extract employed in the transcription reaction (data not shown). Thus, the in vitro transcription system confirms that SRA is a coactivator of MyoD∼E12, and it lends further support to the above-mentioned data that demonstrate that p68/p72 facilitate transactivation by MyoD.

Finally, we assessed the abilities of p68 and SRA to coactivate MyoD in a biologically relevant assay. Expression of MyoD can convert C3H10T1/2 mouse embryonic fibroblasts into skeletal muscle (Tapscott, 2005). C3H10T1/2 cells were transfected with different combinations of MyoD, p68, and SRA expression vectors, and their myogenic conversion was scored by immunostaining with myosin heavy chain (MHC) antibodies and by evaluating MHC gene expression by RT-PCR. As expected, neither p68 nor SRA induced myogenic conversion (data not shown); however, MyoD was effective in conferring the skeletal myogenic phenotype to C3H10T1/2 cells, as indicated by MHC^{+} reactivity and cell morphology. Coexpression of either SRA, p68, or, more effectively, p68 and SRA increased the ability of MyoD to convert C3H10T1/2 cells to muscle (Figures 2G and 2H). Cells expressing p68 and MHC were also always MyoD positive (data not shown). The possibility that transfected p68 and/or SRA transactivated the plasmid expressing exogenous MyoD was ruled out (Figure 2H). Thus, p68 and SRA can effectively increase MyoD-induced conversion of mouse fibroblasts to skeletal muscle cells.

p68 Is Recruited at the Chromatin Regulatory Regions of Actively Transcribed Muscle Loci

To evaluate whether the location of endogenous p68 protein is consistent with its ability to influence muscle gene expression, we used chromatin immunoprecipitation (ChIP) to assay for its presence at two regulatory regions of muscle-specific genes. Chromatin obtained from either undifferentiated myoblasts or differentiated myotubes was immunoprecipitated with an anti-p68 antibody or control immunoglobulin, and the immunoprecipitated DNA was analyzed by RT-PCR with primers amplifying regulatory regions of the MHCIIb and MCK genes. We were not able to obtain a p72 antibody suitable for ChIP and have limited our analysis to p68. Despite being expressed at comparable levels in undifferentiated and differentiated skeletal muscle cells (Figure 3A), p68 was found at muscle regulatory regions exclusively in differentiated myotubes (Figure 3B, MT), when both the MHCIIb and MCK are transcribed (Figure 3C). p68 did not associate with the promoter of the β-globin gene, which is not expressed in skeletal muscle cells. On the contrary, p68 was recruited at the promoter of the estrogen-responsive pS2 gene in MCF7 cells treated with estradiol (Metivier et al., 2003) with an efficiency comparable to that observed for muscle regulatory regions (Figure S2D). p68 could not be detected at the GAPDH promoter either in myoblast or myotubes (Figure S2E). Collectively, these findings indicate that p68 is not a general transcriptional coactivator, but that, rather, it is engaged at regulatory regions of specific genes where its presence is associated with gene activation. To investigate whether p68 was confined to the promoter regions or, after initial promoter recruitment, might become part of the elongation complex, we performed ChIP experiments with antibodies against p68, RNA polymerase II (Pol II), MyoD, or control IgG. The immunoprecipitated chromatin was amplified with primers spanning different regions of the MHCIIb gene corresponding to the promoter, initial coding regions, and coding regions located 19 Kb from the promoter (Figure 3D). While MyoD, p68, and Pol II could be detected at the MHCIIb promoter, only Pol II was present at the MHCIIb coding regions. These results indicate that engagement of p68 is limited to the regulatory chromatin regions of transcribed genes.

p68, p72, and SRA Are Required for Proper Muscle Gene Expression and Skeletal Muscle Differentiation

We reduced the p68/p72 protein levels by RNA interference in C2C12 cells and mouse primary skeletal myoblasts to evaluate their role in regulating muscle gene expression and cell differentiation. Muscle cells were transduced with either a control or a retrovirus expressing a short hairpin (sh) RNA targeting both p68 and p72 RNAs. Cells receiving the shRNA p68/p72 retrovirus had reduced levels of p68/p72 proteins of MHC and failed to form multinucleated myotubes (Figures 4A, 4B, 4E, and 4F). Several experiments were performed to address the specificity of the shRNA p68/p72-induced phenotype. C2C12 cells transfected with chemically modified short interfering (si) double-stranded RNAs, which do not activate the PKR interferon response pathway, corresponding to p68/p72 mRNA sequences also failed to appropriately differentiate (Figure S3A). On the contrary, transfection of siRNAs with "scrambled" p68/p72 sequences did not appreciably affect cell differentiation (Figure S3A). Moreover, the differentiation defects induced by siRNA p68/p72 in C2C12 were rescued by retroviral expression of a p68 bearing three silent third-codon point mutations within the region targeted

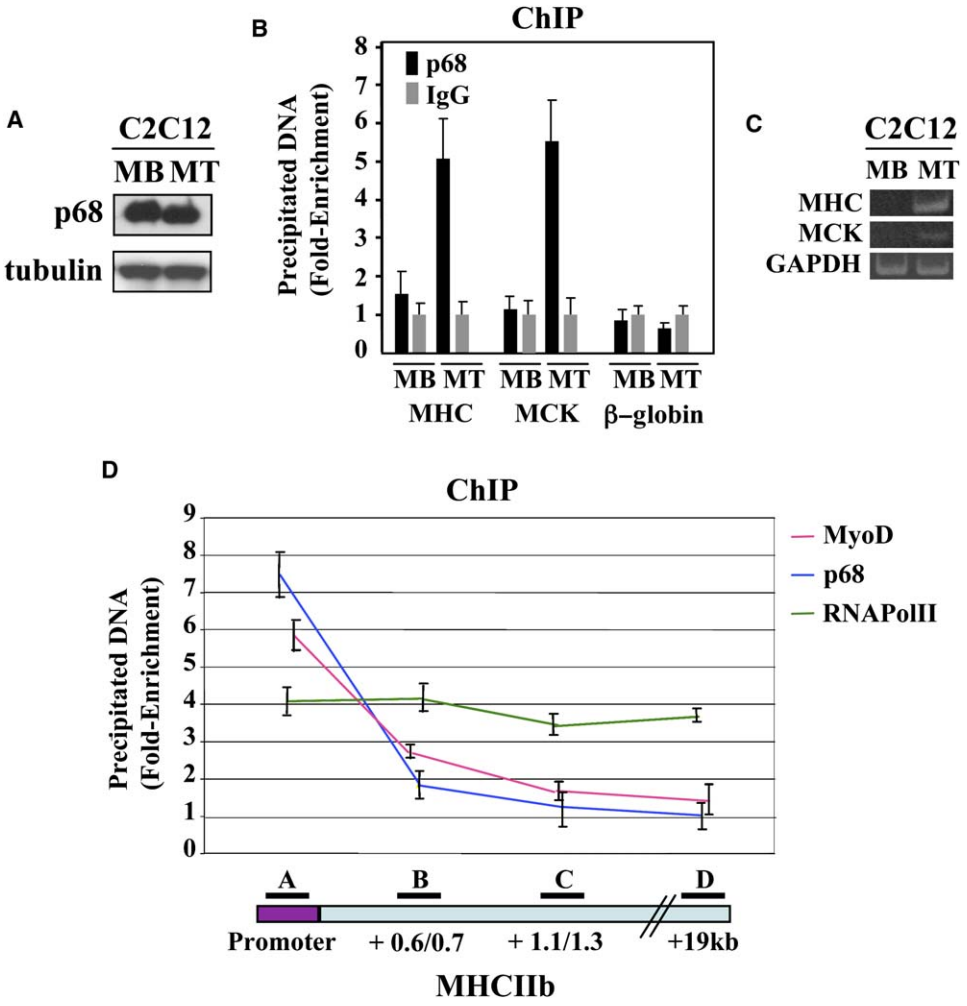

Figure 3. p68 Is Recruited at the Regulatory Regions of Transcriptionally Active Muscle Loci

(A) Immunoblotting of cell extracts from undifferentiated (MB, myoblasts) and differentiated (MT, myotubes) C2C12 cells with p68 and tubulin antibodies.

(B) ChIP assay with chromatin from C2C12 MB or MT and p68 antibodies or control IgG. The precipitated DNA was amplified by RT-PCR with primers for the MHCIIb promoter, MCK enhancer, and β-globin promoter. Error bars represent standard deviations.

(C) RT-PCR of MHCIIb, MCK, and GAPDH transcripts in undifferentiated (MB) or differentiated (MT) C2C12 cells.

(D) ChIP assay of chromatin from differentiated C2C12 cells and antibodies against MyoD, p68, Pol II, and control IgG. Regions corresponding to the promoter (region A), initial coding regions (regions B and C), and coding regions located 19 Kb from the MHCIIb promoter (region D) were amplified by RT-PCR. Error bars represent standard deviations.

by the siRNA p68/72 that render it refractory to RNA interference (Figures S3B and S3C). The inability of shRNA p68/p72 cells to exit the cell cycle and differentiate coincided with a sustained ability to incorporate BrdU; failure to downregulate cyclins D1, E2, A2, B1, and F; and elevated E2F activity when cultured in differentiation medium (Figure S4 and Table S1). To obtain a global description of the genes regulated by p68/p72 in skeletal muscle cells, we employed RNAs derived from shRNA p68/p72 and control C2C12 cells to perform genome-wide expression profiling. The levels of numerous transcripts, including those corresponding to muscle structural proteins (myosins, actins, troponins), muscle enzymes, and transcriptional regulatory proteins, were reduced in C2C12 shRNA p68/p72 cultured in differentiation medium (Table 1, Figure S5, and Expression Profiling Data at http://pepr.cnmcresearch.org). Consistent with a role of p68/p72 in coregulating other transcription factors, non-muscle-specific transcripts were also affected by p68/p72 (Table 1 and http://pepr.cnmcresearch.org). Of interest, the transcripts for two antagonists of the promyogenic Wnt proteins—secreted Frizzled-related sFRP1 and sFRP2 (Leyns et al., 1997)—were increased in C2C12 shRNA p68/p72 cultured in growth medium by 3.9- and 9.6-fold, respectively (http://pepr.cnmcresearch.org). Expression of some of the transcripts identified as being affected by shRNA p68/p72 in the genome-wide profiling was independently evaluated by nonquantitative as well as quantitative PCR (Figures 4C, 4D, and 4G), and the results were found to be concordant with those obtained with the DNA microarray experiments. Previous work has identified MEF2A (Bergstrom et al., 2002), pRb (Bergstrom et al., 2002; Magenta et al., 2003), and follistatin (Iezzi et al., 2004) as genes regulated by MyoD. Interestingly, expression of MEF2A, pRb, and follistatin was

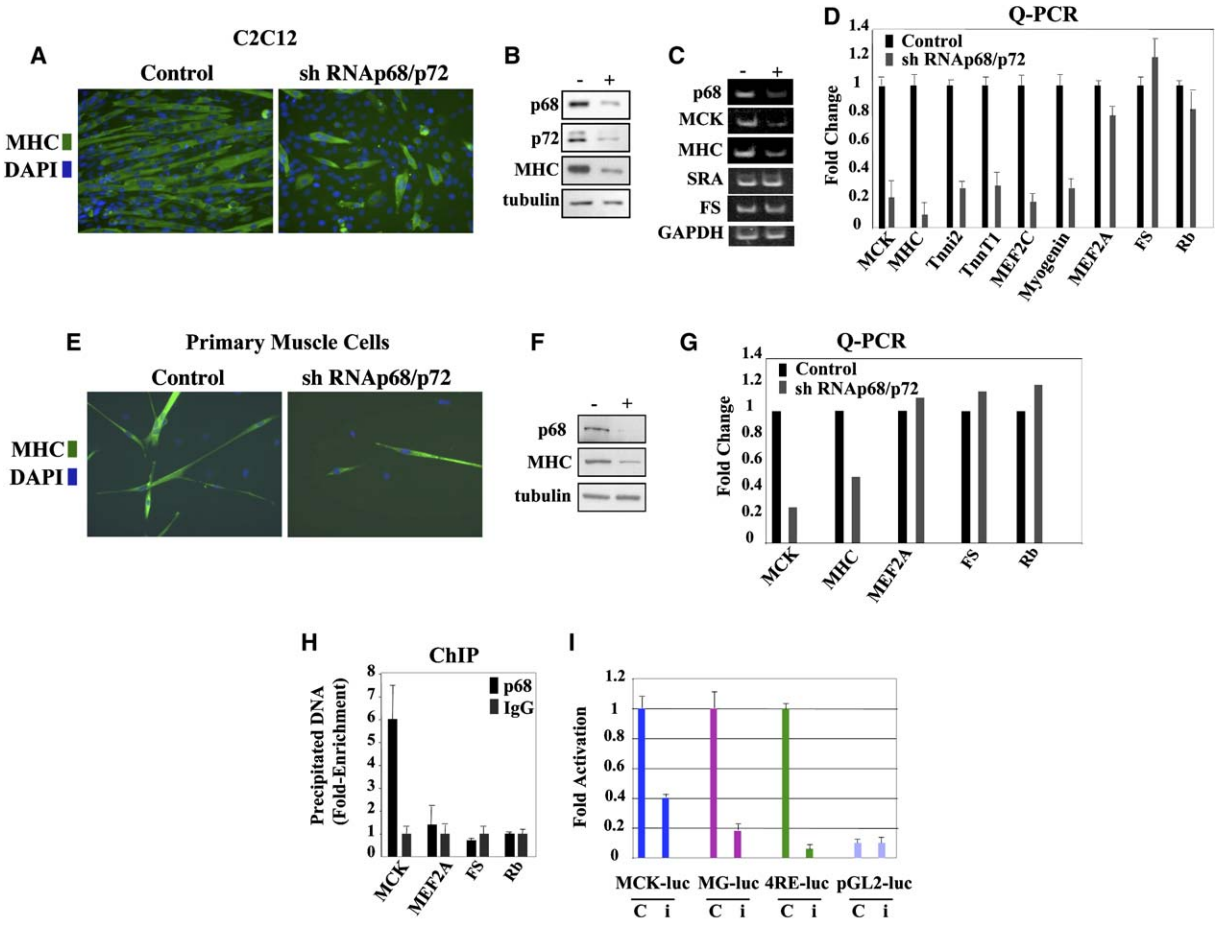

Figure 4. RNA Interference of p68/p72 Impairs Muscle Gene Expression and Skeletal Muscle Differentiation

(A) C2C12 cells transduced with either a control or a retrovirus expressing a short hairpin RNA targeting both p68 and p72 RNAs (shRNA p68/p72). Cells were grown in differentiation medium for 36 hr. Immunofluorescence was performed with an MHC antibody.

(B) Immunoblot analysis of p68, p72, MHC, and tubulin in control (−) and shRNA p68/p72 (+) C2C12 cells.

(C) RT-PCR analysis of p68, MCK, MHC, SRA, FS (follistatin), and GAPDH transcripts in control (−) and shRNA p68/p72 (+) C2C12 cells.

(D) RT-PCR analysis of MCK, MHC, Tnni2 (troponin I2), TnnT1 (troponin T1), MEF2C, myogenin, MEF2A, FS, and Rb (retinoblastoma) transcripts in control and shRNA p68/p72 C2C12 cells. Error bars represent standard deviations.

(E) Mouse primary myoblasts were transduced with either control or shRNA p68/p72 retrovirus, were prompted to differentiated for 36 hr, and were immunostained for MHC.

(F) Immunoblot of p68, MHC, and tubulin of control (−) and shRNA p68/p72 (+) mouse primary myoblasts.

(G) RT-PCR analysis of MCK, MHC, MEF2A, FS, and Rb transcripts in control and shRNA p68/p72 mouse primary myoblasts.

(H) ChIP assay of chromatin from differentiated C2C12 cells and p68 antibody or control IgG. Regulatory regions of the MCK, MEF2A, FS, and Rb genes were amplified by RT-PCR. Error bars represent standard deviations.

(I) The MCK-luc, myogenin (MG)-luc, 4RE-luc, or empty vector pGL2-luc constructs were transfected in control (C) or in shRNA p68/p72 C2C12 (i). Cells were cultured in differentiation medium for 36 hr and were processed to detect for luciferase activity. The CMV-lacZ-expressing vector was cotransfected as an internal control. Error bars represent standard deviations.

increased in differentiated myotubes compared to undifferentiated myoblasts, irrespective of whether p68/p72 was interfered or not (http://pepr.cnmcresearch.org). These results were independently confirmed by quantitative PCR (Figures 4D and 4G). If p68/p72 do not regulate expression of MEF2A, pRb, and follistatin genes, it is possible that these proteins may not be recruited at their chromatin regulatory regions. To test this hypothesis, we performed ChIP experiments and found that, in contrast to the p68/p72-regulated MCK gene, p68 could not be detected on the chromatin regions regulating expression of MEF2A, pRb, and follistatin (Figure 4H). Muscle-specific regulatory regions comprise arrays of several DNA modules recognized by different transcription factors. While the data obtained with an in vitro transcription assay data suggest that the p68/p72 proteins are bona fide coactivators of MyoD-dependent transcription (Figure 2F), we wished to further test whether they behave in a similar fashion in a cellular context. To this end, control and shRNA p68/p72 C2C12 cells were transfected with two reporter constructs bearing naturally occurring muscle regulatory regions—the MCK-luc and the myogenin promoter (MG-luc)—and an artificial construct—the 4RE-luc—bearing four multimerized MyoD binding sites, respectively. The empty cloning vector (pGL-2-luc) was employed as a negative control. The variability of transfection efficiency was corrected by employing a CMV-lacZ vector as an

Table 1. Genes Affected by p68/p72

Nucleotide	UniGene	Muscle Structural Genes	Fold Reduction
NM_008664	Mm.272115	myomesin 2 (*Myom2*)	−7
NM_010855	Mm.297382	myosin, heavy polypeptide 4, skeletal muscle (*Myh4*)	−5
NM_010867	Mm.4103	myomesin 1 (*Myom1*)	−4.3
NM_021503	Mm.141157	myozenin 2	−4.2
NM_033268	Mm.37638	actinin alpha 2	−3.7
NM_020033	Mm.143737	ankyrin repeat domain 2 (stretch responsive muscle)	−3.5
NM_013808	Mm.17235	cysteine and glycine-rich protein 3	−2.8
NM_013456	Mm.5316	actinin alpha 3	−2.5
NM_009405	Mm.39469	troponin I, skeletal, fast 2	−2.5
NM_010861	Mm.1529	myosin, light polypeptide 2, regulatory, cardiac, slow	−2.4
NM_010858	Mm.247636	myosin, light polypeptide 4, alkali; atrial, embryonic	−2
NM_007617	Mm.3924	caveolin 3	−2
NM_011620	Mm.350054	troponin T3, skeletal, fast	−2
NM_010858	Mm.247636	myosin light peptide 4 (*Myl4*)	−2
NP_036048	Mm.29733	LIM domain binding 3	−1.8
NM_021285	Mm.1000	myosin, light polypeptide 1, alkali; atrial, embryonic	−1.8
NM_030679	Mm.340132	myosin, heavy polypeptide 1, skeletal muscle, adult	−1.8
NM_016754	Mm.14526	myosin light chain, phosphorylatable (*Mylpf*)	−1.7
NM_009393	Mm.712	troponin C (*Tnncc*)	−1.6
NM_013593	Mm.201606	myoglobin (*Mb*)	−1.6
NM_011618	Mm.358643	troponin T1 (*Tnnt1*)	−1.6
XM_354614	Mm.340090	myosin, heavy polypeptide 3 (*Myh3*)	−1.5
NM_011652	Mm.26579	titin (*Ttn*)	−1.4
		Metabolic Processes	
NM_009731	Mm.90151	aldo-keto reductase family 1, member B7	−10.7
NM_001037939	Mm.213026	bone gamma carboxyglutamate protein 1	−5
NM_011224	Mm.27806	muscle glycogen phosphorylase	−4.3
NM_145953	Mm.28301	cystathionase (cystathionine gamma-lyase)	−4.3
NM_178405	Mm.207432	ATPase, Na+/K+ transporting, alpha 2 polypeptide	−4.2
NM_009943	Mm.43824	cytochrome c oxidase, subunit VI a, polypeptide 2	−3.8
NM_009204	Mm.10661	solute carrier family 2 (facilitated glucose transporter), member 4	−3.5
NM_011710	Mm.38433	tryptophanyl-tRNA synthetase	−3.5
NM_009802	Mm.232523	carbonic anhydrase 6	−3.4
NM_172451	Mm.384000	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 6	−3.2
NM_013820	Mm.255848	hexokinase 2	−3.2
NM_178405	Mm.207432	ATPase, Na+/K+ transporting, alpha 2 polypeptide	−3
NM_019578	Mm.30978	exostoses (multiple)-like 1	−2.8
NM_144554	Mm.276018	induced in fatty liver dystrophy 2	−2.8
NM_018870	Mm.219627	phosphoglycerate mutase 2	−2.6
NM_013703	Mm.4141	very low-density lipoprotein receptor	−2.6
NM_009944	Mm.12907	cytochrome c oxidase, subunit VIIa 1	−2.6
NM_007504	Mm.35134	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	−2.5
NM_013755	Mm.6375	glycogenin 1	−2.3
NM_007421	Mm.3440	adenylosuccinate synthetase, muscle	−2.2
NM_010514	Mm.3862	insulin-like growth factor 2	−2.1
NM_027807	Mm.218910	cullin 5	−2.1
NM_009710	Mm.261071	ADP-ribosyltransferase 1	−2
NM_016808	Mm.272770	ubiquitin-specific protease 2	−2
NM_007710	Mm.2375	creatin kinase (*Ckm*)	−2
NM_016808	Mm.272770	ubiquitin-specific peptidase 2 (*Usp2*)	−1.9
NM_009722	Mm.321755	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	1.9
		Neurophysiological Processes	
NM_145526	Mm.41252	purinergic receptor P2X, ligand-gated ion channel, 3	−5
NM_009109	Mm.226037	ryanodine receptor 1, skeletal muscle	−3.25
NM_031173	Mm.41252	calcium channel, voltage-dependent, beta 1 subunit	−2.1
NM_008076	Mm.6227	gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2	−2.1
NM_009604	Mm.2810	cholinergic receptor, nicotinic, gamma polypeptide	−2
NM_009497	Mm.28643	vesicle-associated membrane protein 2	−1.8
XM_149971	Mm.374440	G protein-coupled receptor, family C, group 5, member C	−1.8
NM_009604	Mm.2810	cholinergic receptor, nicotinic, gamma polypeptide	−1.7
		Transcription and Chromatin Regulation	
NM_025282	Mm.24001	myocyte enhancer factor 2C	−6.3
NM_013468	Mm.10279	ankyrin repeat domain 1 (cardiac muscle)	−4
NM_019738	Mm.18742	nuclear protein 1 (*Nupr1*)	−3
NM_007837	Mm.110220	DNA damage-inducible transcript 3	−3
NM_031189	Mm.16528	Myogenin[a]	−3

(*Continued on next page*)

Table 1. *Continued*

		Transcription and Chromatin Regulation	
NM_009762	Mm.234274	SET and MYND domain containing 1	−2.7
NM_024250	Mm.46750	PHD finger protein 10	−2.2
NM_009326	Mm.232523	transcription elongation factor A (SII) 1	−1.9
		Signal Transduction	
NM_010200	Mm.7995	fibroblast growth factor 13	−2.9
NM_175540	Mm.189270	ectodysplasin A2 isoform receptor	−2.4
XM_284144	Mm.6256	frizzled homolog 9 (*Drosophila*)	−2
NM_054071	Mm.35691	fibroblast growth factor receptor-like 1	−2
		Miscellaneous	
NM_019581	Mm.22147	GTP binding protein 2	−4.3
NM_013549	Mm.205601	histone 2, H2aa1	−4
NM_007836	Mm.72235	growth arrest and DNA damage-inducible 45 alpha	−3.7
NM_010174	Mm.22220	fatty acid binding protein 3, muscle and heart	−3.6
NM_015786	Mm.193539	histone 1, H1c	−3.3
NM_023422	Mm.261676	histone 1, H2bc	−3.3
NM_019581	Mm.22147	GTP binding protein 2	−3.2
NM_153502	Mm.41421	ankyrin repeat domain 23	−2.5
NM_013868	Mm.46181	heat shock protein family, member 7 (cardiovascular)	−2.5
NM_133769	Mm.154358	cytoplasmic FMR1 interacting protein 2	−2.4
NM_007871	Mm.39292	dynamin 2	−2.4
NM_011193	Mm.2534	proline-serine-threonine phosphatase-interacting protein 1	−2.4
NM_013712	Mm.46232	integrin beta 1 binding protein 2	−2.3
NM_145375	Mm.28887	transmembrane 6 superfamily member 1	−2.3
NM_020013	Mm.143736	fibroblast growth factor 21	−2

Total RNA was obtained from C2C12 cells cultured in differentiation medium for 36 hr. The nucleotide and UniGene accession numbers are indicated in the first two columns. Fold reduction indicates the extent of reduction in gene expression observed in shRNA p68/p72 C2C12 cells compared to control cells.
[a] Measured by qRT-PCR after 12 hr of differentiation.

internal control. The results of these experiments (Figure 4I) indicate that cells with reduced levels of p68/p72 failed to appropriately activate the muscle-specific constructs, including the 4RE-luc, further suggesting that p68/p72 are transcriptional coactivators of the myogenic bHLH proteins in skeletal muscle cells.

SRA transcript levels do not change during differentiation (Figure S1C), but the finding that SRA associates with and coactivates MyoD prompted us to further evaluate its role in muscle gene expression. The SRA transcript levels were reduced by transfecting C2C12 cells with in vitro-transcribed and Dicer-processed SRA RNA (Figure 5A). Several attempts to lower SRA by using a retroviral-based shRNA system were unsuccessful. The difficulties we experienced with the retroviral approach may be related to the complex secondary structure of SRA (Lanz et al., 2002) that may prevent access of single siRNAs to specific regions of the RNA target. Cells transfected with two rounds of Dicer-processed SRA RNAs were impaired in their ability to differentiate and to activate transcription of several muscle-specific genes (Figures 5B–5E). The potential nonspecific, off-target effects of the small RNAs were controlled by transfecting Dicer-processed negative control RNAs that did not affect cell differentiation (Figure 5). Similar, to the results obtained with p68/p72 interference, expression of MEF2A, pRb, and follistatin genes was not influenced by SRA (Figure 5E). These effects are unlikely to be mediated by reduced levels of p68 since SRA interference did not affect p68 expression (Figure 5D). Overall, the results of the experiments described in this paragraph indicate that p68/p72 and SRA are required for undifferentiated myoblasts to properly mature into terminally differentiated myotubes, and that p68/p72 are coregulators of myogenic bHLH in skeletal muscle cells. Moreover, they indicate that regulation of a subset of genes activated by MyoD is not influenced by either p68/72 or SRA.

p68 and p72 Regulate the Assembly of Selected Members of the Transcriptome and Are Critical for the Chromatin Remodeling of Muscle Regulatory Regions

p68/p72 regulate transcription mediated by the nuclear hormone receptors p53 (Endoh et al., 1999; Watanabe et al., 2001; Metivier et al., 2003; Bates et al., 2005) and MyoD (this study), but the mechanisms through which they influence transcription have not been defined. Since p68 copurifies with MyoD and is found at chromatin regulatory regions of actively transcribed muscle-specific genes, we considered the possibility that p68/p72 may be involved in the assembly of the muscle transcriptome. MyoD can access genes in repressive chromatin, initiate chromatin remodeling, and activate transcription through recruitment of the ATP-dependent chromatin remodeling SWI/SNF complex and histone acetyltransferase p300 and PCAF (Sartorelli and Caretti, 2005; Tapscott, 2005). Moreover, TBP stabilizes the binding of MyoD to its DNA binding sites and facilitates the association of TFIIB with the preinitation complex (Heller and Bengal, 1998). Therefore, we evaluated the presence of MyoD; p300; the ATPase subunit of the SWI/SNF complex, Brg-1; TBP; Pol II; and p68 at the MHCIIb and troponin C promoters by ChIP assay. Immunoprecipitations with nonspecific IgG were carried out as a negative control. Amplification of the β-globin promoter was employed as a negative control. Surprisingly, we found

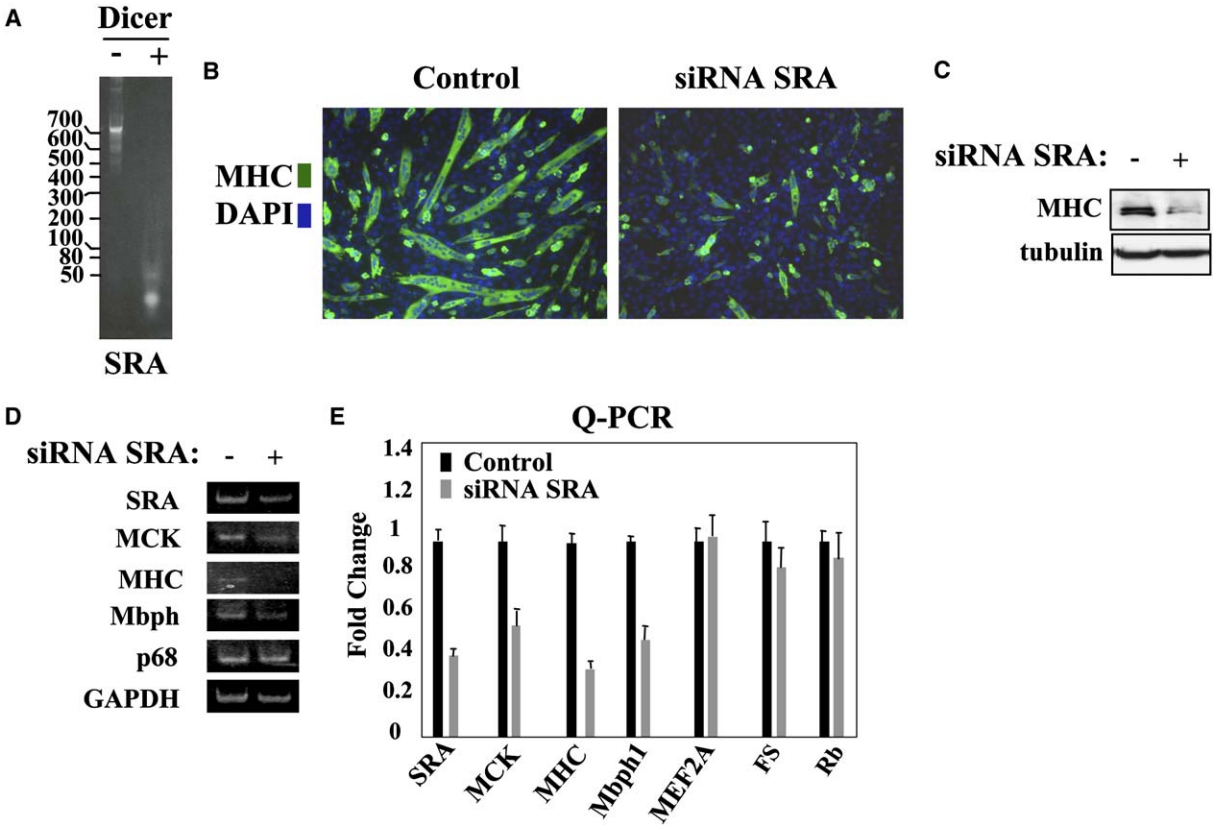

Figure 5. RNA Interference of SRA Impairs Muscle Gene Expression and Skeletal Muscle Differentiation

(A) In vitro-transcribed double-stranded SRA RNA was incubated either in the absence (−) or presence (+) of the Dicer protein and was resolved on a 1% agarose gel.

(B) C2C12 cells were transfected twice with either a control or Dicer-treated SRA RNA. After the second transfection, cells were grown in differentiation medium for 36 hr. Immunofluorescence was performed with an MHC antibody.

(C) Immonoblot analysis of MHC and tubulin in control and siRNA SRA-transfected C2C12 cells.

(D) RT-PCR analysis of SRA, MCK, MHC, Mbph1 (myosin binding protein 1), p68, and GAPDH transcripts in control and siRNA SRA-transfected C2C12 cells.

(E) RT-PCR analysis of SRA, MCK, MHC, Mbph1, MEF2A, FS, and Rb transcripts in control and siRNA SRA-transfected C2C12 cells. Error bars represent standard deviations.

that reducing the levels of p68/p72 proteins impaired the recruitment of Brg-1, TBP, and Pol II without significantly affecting binding of MyoD and p300 (Figures 6A and 6B). Consistent with the unperturbed recruitment of p300, histone H4 acetylation at the MHCIIb and troponin C promoters was not affected in shRNA p68/p72. Expression of FS and Rb is not regulated by p68/p72, and, accordingly, p68 is not recruited at their regulatory regions (Figure 4). Consistent with these observations, reducing the levels of p68/p72 did not affect recruitment of Brg-1, TBP, Pol II, and p300 at the FS and Rb promoters (Figure S6A). Since SWI/SNF has been shown to mediate chromatin remodeling of MyoD-regulated genes (de la Serna et al., 2001; Simone et al., 2004), we probed the chromatin structure of the MHCIIb promoter by restriction endonuclease accessibility. The chromatin of the MHCIIb promoter obtained from shRNA p68/p72 cells was less accessible to the endonuclease BanI when compared to that of control cells, indicating that reduced recruitment of Brg-1 was accompanied by impaired chromatin remodeling (Figures 6D and 6E). No differences in chromatin accessibility were noted in the β-globin gene (Figure 6D). The protein levels of Brg-1, TBP, and Pol II are not diminished in cells with reduced levels of p68/p72 (Figure 6C), and this excludes the possibility that p68/p72 may directly regulate their expression/stabilization. Therefore, we speculated that the mechanism through which the RNA helicases affect chromatin recruitment of Brg-1, TBP, and Pol II might entail formation of a protein complex. To directly test this hypothesis, cells were transfected with a Flag-p68 expression vector, and cellular extracts were prepared and immunoprecipitated with either control IgG or anti-Flag antibody. Immunoblot analysis with anti-Brg-1, -TBP, or -Pol II antibodies revealed that Flag-p68 could associate with endogenous Brg-1, TBP, and Pol II (Figure 6F). To extend these findings to endogenous proteins, immunoprecipitation experiments were conducted with anti-Brg-1, anti-TBP, or anti-Pol II antibodies and extract derived from skeletal muscle cells. Immunoblotting of the precipitated materials with anti-p68 antibodies indicated that endogenous p68 could associate with endogenous Brg-1, TBP, and Pol II (Figure 6G). Since MyoD interacts with Brg-1 (Simone et al., 2004; de la Serna et al., 2005), we asked whether p68/p72 may promote and/or stabilize their association. Indeed, the interaction of Brg-1 with MyoD

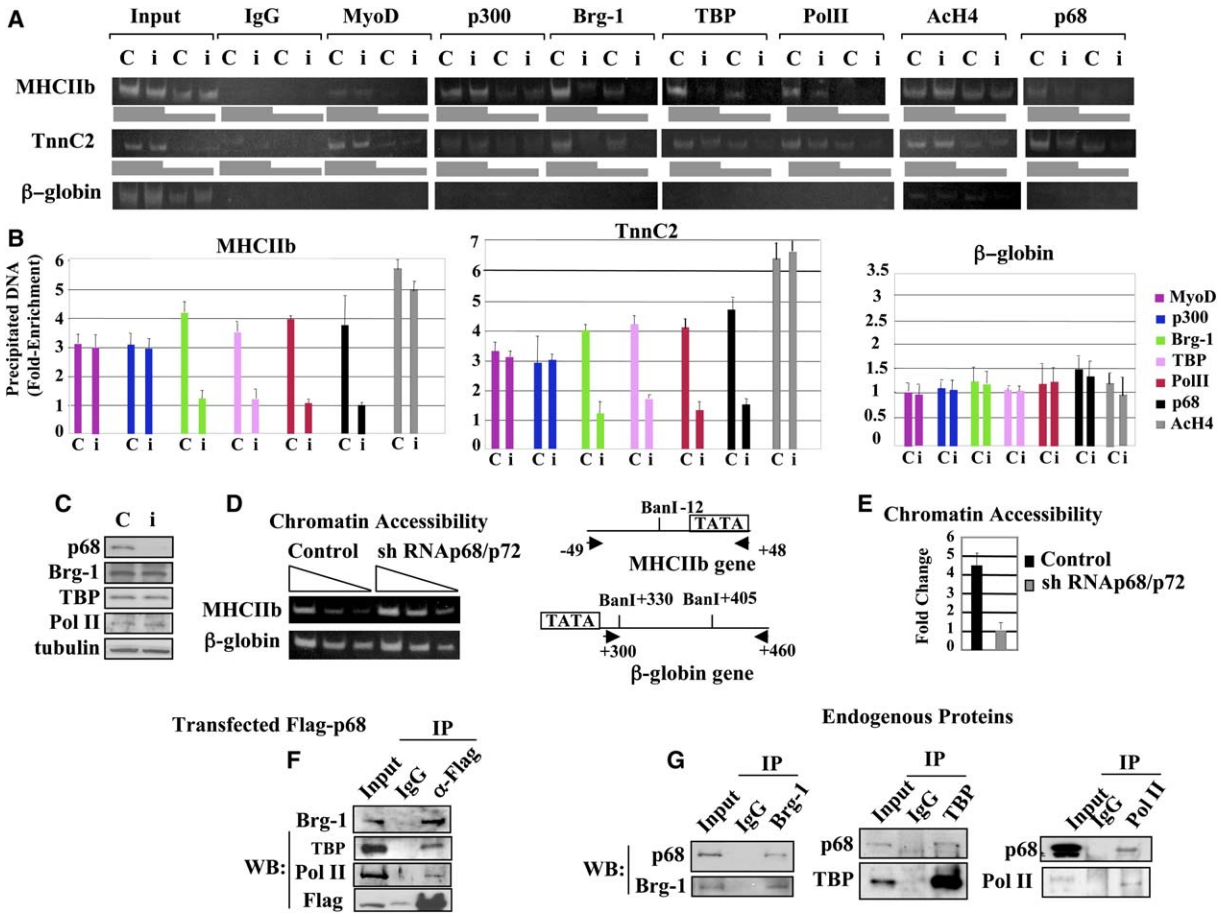

Figure 6. Chromatin Recruitment of Brg-1, TBP, and Pol II at the MHCIIb and TnnC2 Promoters and Chromatin Remodeling at the MHCIIb Promoter Are Impaired in Cells with Reduced Levels of p68/p72

(A) ChIP assay performed with chromatin derived from C2C12 cells transfected with either control scrambled (C) or p68/p72 siRNAs (i = interfered) and grown in differentiation medium for 36 hr. The antibodies employed are indicated at the top of the panel. For each point, two different dilutions of the immunoprecipitated DNA were employed. The immunoprecipitated DNA was amplified by PCR with specific primers for the MHCIIb and fast skeletal troponin C (TnnC2) promoters. The β-globin promoter was amplified as a negative control.

(B) The ChIP assay was performed as described in (A), and DNA amplification was performed by RT-PCR. The fold enrichment was obtained after correcting for the values observed with the DNA derived from immunoprecipitations performed with nonspecific IgG, which were set as one unit in each calculation, as described by Caretti et al. (2004). RT-PCR runs were performed with triplicate samples and were repeated with DNA obtained from three independent experiments. The error bars indicate standard errors of the mean.

(C) Immunoblotting of proteins derived from C2C12 cells transfected with either control scrambled (C) or p68/p72 siRNAs (i) The antibodies employed are indicated on the left part of the panel.

(D) Nuclei derived from either control or shRNA p68/p72 C2C12 cells were isolated and incubated with BanI for 45 min. The DNA was purified, and three dilutions (1:30; 1:100, and 1:300) were amplified by PCR by using oligonucleotides flanking the BanI site of the MHCIIb promoter and, as a control, oligonucleotides amplifying a fragment of the β–globin gene. (Right panel) Schematic representation of the location of the BanI sites in the MHCIIb and β-globin genes. The numbers refer to the start of transcription at +1.

(E) The chromatin endonuclease accessibility assay was performed as described in (D), and DNA amplification was performed by RT-PCR. The fold enrichment was derived after normalization with values obtained from the amplification of the β-globin gene. Error bars represent standard deviations.

(F) 293 T cells were transfected with the Flag-p68 expression vector, and cell extracts were prepared and immunopurified on anti-Flag antibody-conjugated agarose or control IgG. The immunoprecipitated material was analyzed by immunoblotting with antibodies against Brg-1, TBP, Pol II, and Flag.

(G) Cell extracts derived from C2C12 cells were immunoprecipitated with antibodies against Brg-1, TBP, Pol II, or control IgG. The immunoprecipitated material was immunoblotted with antibodies against p68, Brg-1, TBP, or Pol II.

was diminished in extracts derived from C2C12 cells in which levels of p68/p72 were reduced by RNA interference, suggesting that p68/p72 may act as a tether between MyoD and Brg-1 (Figure S6B). Overall, these results indicate that p68/p72 direct and/or stabilize chromatin recruitment of proteins involved in the formation of the transcription initiation complex and chromatin remodeling.

Discussion

We have isolated the prototypic p68 (Ford et al., 1988) and highly related p72 (Lamm et al., 1996) RNA helicases as proteins associated with MyoD. The SRA was also found to be complexed with MyoD. To isolate MyoD-associated polypeptides, we have chosen to express MyoD in HeLa cells for the following reasons: (1) large

amounts of nuclear extracts can be obtained from HeLa cells grown in suspension (Nakatani and Ogryzko, 2003), and (2) the cell cycle-arresting properties of MyoD (Crescenzi et al., 1990) are ineffective in HeLa cells (data not shown). These characteristics have allowed us to obtain sufficient nuclear extracts to be subjected to two consecutive immunoaffinity purification steps. The association of p68/p72 with overexpressed MyoD initially detected in HeLa extracts was confirmed by using cell extracts derived from skeletal muscle cells, and all the subsequent experiments—aimed at the functional characterizations of p68/p72 and SRA—were performed in skeletal muscle cells. Given that only a small fraction of p68 interacted with MyoD in muscle cells, we were surprised to observe that SRA efficiently coimmunoprecipitated with MyoD. These findings are compatible with the existence of complexes containing MyoD and SRA, but not p68. Consistent with a role in regulating muscle gene expression, p68—despite being expressed at equivalent levels in undifferentiated and differentiated muscle cells—was recruited at muscle loci only when these were transcriptionally active. This observation can be explained by the ability of p68 to interact with MyoD and be recruited on the chromatin only when MyoD binds and activates its targets. Muscle gene expression and skeletal muscle differentiation were impaired in cells with reduced levels of either p68/p72 or SRA.

The Noncoding RNA Steroid Receptor Activator and the RNA Helicases p68 and p72

SRA was originally identified as a noncoding RNA highly enriched in liver and skeletal muscle and present in distinct ribonucleoprotein complexes selectively interacting with and activating transcription mediated by steroid hormone receptors (Lanz et al., 1999). Further studies indicated that SRA is subjected to both negative and positive regulations. SRA-mediated steroid receptor transcription activity could be suppressed by a cofactor, SHARP (SMRT/HDAC1-associated repressor protein), whose expression is itself steroid inducible, suggesting that SHARP may be part of a feedback mechanism to attenuate hormonal response (Shi et al., 2001). More recently, another corepressor, the small protein SLIRP, has been demonstrated to bind SRA and to cooperate with SHARP in inducing repression of nuclear receptor transactivation in a SRA-dependent manner (Hatchell et al., 2006). At variance, the pseudouridine synthase mPus1p, an enzyme that isomerizes uridine to pseudouridine in noncoding RNAs, catalyzes pseudouridylation of SRA in vitro. mPus1p cooperates with SRA to enhance not only RAR-dependent transcription, but also that mediated by other nuclear receptors (Zhao et al., 2004). While pseudouridylated SRA has been suggested to assume an "active" conformation, its exact role within a transcriptional complex has not been yet elucidated. Our findings indicate that the role of SRA as a transcriptional coactivator is not confined to the regulation of steroid hormone receptors, suggesting a broader involvement of this noncoding RNA in transcriptional control. We cannot exclude the possibility that SRA, rather than acting as a noncoding RNA, may encode a small endogenous protein. Nonetheless, we do not favor this hypothesis for the following two reasons: (1) our in vitro transcription experiments demonstrating a coactivatory role of SRA were performed with in vitro-transcribed SRA, and (2) the transfection experiments showing that SRA coactivates MyoD were performed with a mouse SRA cDNA expression vector lacking a start codon.

p68/p72 are members of the DEAD-box family of RNA helicases, characterized by a stretch of eight conserved motifs including the Asp-Glu-Ala-Asp (D-E-A-D) sequence. These RNA helicases have been highly conserved through evolution, and members of the DEAD-box family are found in all species, from bacteria to humans. In yeast, members of the DEAD-box family are required for cell growth, and in mammalians they regulate several biological processes, including transcription, splicing, and translation (Tanner and Linder, 2001). A relevant function of p68/p72 may be related to their RNA helicase activity (Tanner and Linder, 2001). However, the helicase activity of p68 is dispensable to assist transcription mediated by MyoD (Figure 2) and by other activators (Endoh et al., 1999; Bates et al., 2005). Therefore, p68 may have additional functions relevant for transcriptional regulation that have remained elusive.

Role of p68 and p72 Proteins in Directing the Assembly of the Transcriptome and Chromatin Remodeling

Our findings indicate that p68/p72 regulate formation of a competent transcriptional complex. Reducing the protein levels of p68/p72 through RNA interference reduced chromatin recruitment not only of p68, but also of the TATA binding protein TBP, Pol II, and the brahma-related gene-1 Brg-1, the catalytic subunit of the ATPase chromatin remodelling SWI/SNF complex, without affecting their protein levels. As a consequence, formation of the transcription initiation complex at the MHCIIb and troponin C promoters was impaired (as revealed by reduced recruitment of Pol II), and nucleosome remodeling was ineffective (as indicated by inefficient chromatin accessibility to restriction enzymes). We propose a model in which p68/p72 actively recruit and/or stabilize the chromatin interactions of members of the muscle transcriptome by physically interacting with Brg-1, TBP, and Pol II. p68/p 72 are ubiquitously expressed and regulate transcription mediated by other activators (Endoh et al., 1999; Watanabe et al., 2001; Metivier et al., 2003; Bates et al., 2005), and it is possible that the same steps regulated by p68/p72 during transcriptional activation in muscle cells may also be affected in other transcriptional systems. Nonetheless, it is important to note that the transcriptional requirements for p68/p72 (and SRA) are not absolute, even within genes regulated by MyoD (Figures 4 and 5). These findings suggest a specific function of p68/p72 in regulating discrete gene programs. Finally, the existence of a functional relationship between p68/p72 and chromatin remodeling complexes is suggested by the observation that genes whose expression was affected by p68/p72, such as MCK, MHC, myogenin, Tnni2, and MybpH, require Brg-1 (de la Serna et al., 2005), whereas those not affected by p68/p72, such as pRb, MEF2A, cyclin D3, Hes6, MyoD, and Chrnb1 (Figures 4C, 4D, and 4G and http://pepr.cnmcresearch.org), are either not (pRb, cyclin D3, Hes6, MyoD) or marginally (MEF2A and Chrnb1) affected by Brg-1 (de la Serna et al., 2005). The selective recruitment of interacting proteins and functional RNAs at discrete subsets of

genes may be part of an evolutionary strategy employed by MyoD to generate temporal and gene-specific activation (Tapscott, 2005).

Experimental Procedures

Plasmids and Retroviral Constructs

The constructs are described in Supplemental Data.

MyoD-Expressing Cells and Protein Purification

HeLa cells were virally transduced and purified by three rounds of affinity cell sorting with magnetic beads coated with IL2R antibodies as described (Nakatani and Ogryzko, 2003). Flag-HA-MyoD was immunoprecipitated and purified from nuclear extracts of HeLa cells expressing MyoD as described by Nakatani and Ogryzko (2003).

Mass Spectrometry

Mass spectrometry was performed at the Harvard Microchemistry Facility (HMF) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS) on a Finnigan LCQ DECA quadrupole ion trap mass spectrometer. MS/MS spectra were correlated with known peptide sequences by using the Sequest algorithm and programs developed at the HMF.

siRNAs

The pcDNA3-mSRA and the pcDNA-AS-mSRA plasmids were linearized with XhoI and were used to prepare a population of siRNA by using the Silencer siRNA Cocktail Kit (Dicer) (Ambion) by following the manufacturer's instructions. The negative control siRNA cocktail was prepared from the β-gal gene. Transfection of mSRA and negative control siRNA was performed as described (Caretti et al., 2004). C2C12 cells were transfected with a mixture of p68/p72 duplex Stealth (Invitrogen) siRNA or their corresponding scrambled siRNA (100 nmol each) (Supplemental Data) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions; after 30 hr, cells were allowed to differentiate for 36 hr and were harvested.

Chromatin Immunoprecipitation Assay and Chromatin Endonuclease Accessibility Assay

The methods are reported in Supplemental Data.

Immunoprecipitation and RT-PCR

The methods are reported in Supplemental Data.

Cell Culture and shRNA

Retroviruses expressing p68/p72-specific short hairpin RNA and control retroviruses were generated by transient transfection of the SUPER.Retro.Puro sh p68/p72 or pSUPER.Retro.Puro vectors in Phoenix cells. The viral supernatants were employed to transduced C2C12 cells. Cells were selected with 2 μg/ml puromycin and then cultured in either growth medium (DMEM supplemented with 20% fetal calf serum, 1% antibiotics) or differentiation medium (DMEM supplemented with 2% horse serum, 1% antibiotics) for 36 hr.

Transient Transfections

Transient transfections of NIH3T3, control and shRNA p68/p72 C2C12 cells, luciferase, and β-gal assays were performed as previously described (Caretti et al., 2004).

Expression Profiling

The methods are described in Supplemental Data. Gene lists at each time point are available at the Public Expression Profiling Resource (http://pepr.cnmcresearch.org, tissue types "CELLS" project "VSartorelli p68 SMC differentiation").

Immunoprecipitation, Immunoblotting, and Immunofluorescence

The methods are reported in Supplemental Data.

Protein Purification, Chromatin Assembly, and In Vitro Transcription Assay

Flag-MyoD∼E12, Flag-PCAF, His-p300, Flag-p68, and Flag-p72 proteins were expressed in baculovirus-infected Sf-9 cells and affinity purified with M2-agarose resin (Sigma) as previously described (Dilworth et al., 2004). SRA was in vitro transcribed and purified by using the Mega Script T7 kit (Ambion). Nucleosomal arrays were generated on the 4R-MCK-pAL3 and pG1 templates by using histones purified from HeLa cells and recombinant NAP-1 and ACF complex as previously described (Ito et al., 1999). Flag-MyoD∼E12 (1 pmol), Flag-PCAF (25 fmol), His-p300 (25 fmol), and Flag-p68, Flag-p72, and SRA (25 fmol of each) were added to reactions as indicated and incubated with 15 fmol nucleosomal 4R-MCK-pAL3 or pG1 template in the presence of HeLa nuclear extract (60 μg) and acetyl CoA (1 μM). RNA recovered from the reactions was subjected to S1 nuclease analysis as previously described (Dilworth et al., 2004).

Oligonucleotides

The oligonucleotide sequences are reported in Supplemental Data.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, figures, and a table and are available at http://www.developmentalcell.com/cgi/content/full/11/4/547/DC1/.

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