Regulation of MyoD Activity and Muscle Cell Differentiation by MDM2, pRb, and Sp1*

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Muscle cell differentiation is controlled by a complex set of interactions between tissue restricted transcription factors, ubiquitously expressed transcription factors, and cell cycle regulatory proteins. We previously found that amplification of MDM2 in rhabdomyosarcoma cells interferes with MyoD activity and consequently inhibits overt muscle cell differentiation (1). Recently, we found that MDM2 interacts with Sp1 and inhibits Sp1-dependent transcription and that pRb can restore Sp1 activity by displacing MDM2 from Sp1 (2). In this report, we show that forced expression of Sp1 can restore MyoD activity and restore overt muscle cell differentiation in cells with amplified MDM2. Furthermore, we show that pRb can also restore MyoD activity and muscle cell differentiation in cells with amplified MDM2. Surprisingly, we found that the MyoD-interacting domain of pRb is dispensable for this activity. We show that the C-terminal, MDM2-interacting domain of pRb is both necessary and sufficient to restore muscle cell differentiation in cells with amplified MDM2. We also show that the C-terminal MDM2-interacting domain of pRb can promote premature differentiation of proliferating myoblast cells. Our data support a model in which the pRb:MDM2 interaction modulates Sp1 activity during normal muscle cell differentiation.

We previously found that amplification of MDM2 in rhabdomyosarcoma cells inhibits MyoD function and inhibits muscle cell differentiation (1). The oncogenic properties of MDM2 are thought to result from interactions with several cell cycle regulatory proteins. MDM2 interacts directly with the tumor suppressor protein p53 (3) and blocks p53-mediated transcription (4–9). In addition, MDM2 has been shown to target p53 for rapid degradation (10, 11). MDM2 also interacts with a second tumor suppressor protein, the retinoblastoma-associated protein, pRb. This MDM2-pRb interaction results in inhibition of pRb growth regulatory function (12, 13). Furthermore, MDM2 interacts with the activation domains of the S-phase-promoting transcription factors E2F1 and DP1, resulting in stimulation of E2F1/DP1 transcriptional activity (14). Taken together, these observations suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRb but also promotes the G1-to-S-phase transition by stimulating E2F1/DP1 activity. The results presented here show that MDM2 can also modulate cellular differentiation through pRb and Sp1.

Differentiating muscle cells fuse to form multinucleated myotubes, thereby withdrawing permanently from the cell cycle. This process is controlled by the MyoD family (MyoD, Myf-5, myogenin, and MRF4/Myf-6) of muscle-specific transcription factors (15). The MyoD family of basic helix-loop-helix transcription factors acts at multiple points in the myogenic lineage to establish muscle cell identity and control terminal differentiation. MyoD is found in a multiprotein complex that contains tissue-restricted (SRF or MEF2C) and ubiquitously expressed (E12/E47 and Sp1) transcription factors (16). Furthermore, interaction between MyoD and Sp1 has been shown to be critical for the expression of many muscle-specific genes (16, 17). We recently showed that MDM2 inhibits transcriptional activation of Sp1 by binding to its C-terminal DNA binding domain and that pRb can counteract this inhibition by displacing Sp1 from MDM2 (2). In this report we show that forced expression of either pRb or Sp1 can restore MyoD activity and muscle cell differentiation in cells with amplified MDM2.

**EXPERIMENTAL PROCEDURES**

**Cells and Constructs—**C2C12 and NIH3T3 were from American Type Culture Collection. Mdm2/p53 null cells were from Dr. S. Jones. Mdm2 over-expressing cells C2C12/R1811 were generated in the laboratory by microcell-mediated chromosome transfer. DHFRLUC was from the laboratory of Dr. G. Merrill. CMV-MDM2 was from Dr. B. Vogelstein and Rb wild type from Dr. W. Kaelin. CMV-Sp1 was a gift from Dr. Tjian. CMV-E2F was generated by Dr. K. Helin. Rb N terminus and C terminus with FLAGs were generated in by Daniel Stauffer with PCR and subcloning into pcDNA3.

Transfections and Luciferase Assay—NIH3T3 cells were transfected with LipofectAMINE (Invitrogen), and C2C12 and C2C12/R1811 were transfected with LipofectAMINE Super 2000 for better transfection efficiency. A luciferase assay was done after cells were lysed with Triton lysis buffer with 1 mM dithiothreitol. 5–10 μl of total cell lysates was used to measure activity levels with a luminometer. The results were shown either as side-by-side duplicates or as an average of four individual data with error bars.

**Differentiation Assay—**C2C12 and C2C12/R1811 were transfected with Rb, Sp1, or an empty vector as control. 24 h later, cells were switched to 2% serum, and culture was continued in the medium for 3 days. Cells were lysed with Nonidet P-40 buffer for Western blot analysis or fixed with 4% paraformaldehyde for immunocytochemistry. For some experiments, C2C12 were transfected with Rb or Sp1. The next day, fresh medium with 15% fetal bovine serum was added, and cells were cultured in the medium for 3 day with medium changed daily.

Western Blot and Immunoprecipitation—Whole cell lysates were prepared by lysis in nuclear extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 plus protease inhibitors). Anti-E2F and anti-Sp1 was purchased from Upstate Biotechnology and anti-Mdm2 from

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†The abbreviations used are: MDM2, mouse double minute gene 2; GST, glutathione S-transferase; CMV, cytomegalovirus.
Santa Cruz Biotechnology. Anti-myosin heavy chain (MHC) MF-20 was from the hybridoma bank of the University of Iowa. The primary antibodies were diluted in TBST blocking buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 0.1% Tween 20) with 5% bovine serum albumin and incubated on a rocker for 4 h at room temperature or overnight in a cold room. The secondary antibodies were diluted to 1:10,000 and incubated at room temperature for 1 h. For immunoprecipitation, 500 μg of whole cell lysis was incubated with 2 μg of antibodies overnight in a cold room and then bound to protein-A/G beads (Santa Cruz Biotechnology) for 2 h before washing and loading onto 10% SDS gel.

**Immunocytochemistry**—For immunocytochemistry studies, cells were fixed in 4% paraformaldehyde for 15 min, washed with PBS three times, and then fixed in 1:1 methanol and acetone for 2 min. Cells were then washed with PBS three times, blocked with 2% lamb serum for 1 h, incubated with anti-MHC at 1:1000 overnight in a cold room, and then washed with PBST three times and incubated with goat anti-mouse IgG conjugated with Alexa Fluor 594 (Molecular Probes) for 2 h. Cells were counted under a fluorescence microscope.

**GST Pull-down**—Glutathione S-transferase fusion proteins were generated in BL-21 cells, induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and released by sonication and digestion in 1% Triton X-100. GST-Rb was cleaved with thrombin, whereas GST-Sp1 was affinity-purified using glutathione-agarose beads (Sigma). Protein concentrations were determined by DC protein assay kit (Bio-Rad). In vitro translated [35S]methionine (PerkinElmer Life Sciences)-labeled E2F1 was prepared using a Promega Tnt kit. Proteins were incubated with GST affinity-purified fusion Sp1 proteins for 2 h at 4°C with constant agitation. After extensive washes with TENN buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40) and radioimmune precipitation buffer, bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE. E2F1 was detected by Western blot analysis using a polyclonal anti-E2F antibody followed by a horseradish peroxidase-labeled goat anti-rabbit IgG antibody and detection with ECL reagents.

**RESULTS**

We previously identified a derivative rhabdomyosarcoma chromosome capable of inhibiting overt muscle cell differentiation when introduced, via microcell-mediated chromosome transfer, into the mouse myoblast cell line C2C12. This derivative chromosome contains a region of amplified DNA originating from chromosome 12q13–14. Testing the amplified genes for the ability to inhibit muscle-specific gene expression indicated that over-expression of MDM2 interferes with MyoD function and consequently inhibits muscle differentiation (1).

**FIG. 1.** *Loss of MyoD activity and muscle cell differentiation in cells with amplified MDM2.* A, C2C12 and a microcell hybrid containing amplified MDM2 (MDM2+) were cultured in differentiation media (1% serum) for 3 days and stained with an antibody against myosin heavy chain (1). B and C, Western blot analysis on C2C12 and a microcell hybrid containing amplified MDM2 probed with antibodies against MDM2, γ-tubulin, and MyoD. D, transactivation activity of MyoD in cells with amplified MDM2. C2C12 cells and a hybrid with amplified MDM2 were transfected with the synthetic MyoD reporter construct 4RTKCAT and increasing amounts of a MyoD expression vector. RSVCAT was used to control for transfection efficiency between cell lines.

Fig. 1. Loss of MyoD activity and muscle cell differentiation in cells with amplified MDM2. A, C2C12 and a microcell hybrid containing amplified MDM2 (MDM2+) were cultured in differentiation media (1% serum) for 3 days and stained with an antibody against myosin heavy chain (1). B and C, Western blot analysis on C2C12 and a microcell hybrid containing amplified MDM2 probed with antibodies against MDM2, γ-tubulin, and MyoD. D, transactivation activity of MyoD in cells with amplified MDM2. C2C12 cells and a hybrid with amplified MDM2 were transfected with the synthetic MyoD reporter construct 4RTKCAT and increasing amounts of a MyoD expression vector. RSVCAT was used to control for transfection efficiency between cell lines.

Inhibition of MyoD Activity by MDM2—To begin to determine the mechanism by which MDM2 interferes with MyoD function, we assayed MyoD-dependent transactivation on the muscle-specific enhancer/promoter construct MCKCAT and on the MyoD-dependent reporter 4RTKCAT (contains four copies of the high affinity MyoD binding site from the MCK enhancer upstream of the herpesvirus-thymidine kinase promoter). Fig. 2A shows that co-transfection of increasing amounts of an

2 T. A. Fiddler and M. J. Thayer, unpublished observations.
MDM2 expression vector results in inhibition of MyoD-dependent transactivation. As a control, we assayed p53-dependent transactivation on the p21 promoter; increasing the amount of MDM2 expression vector resulted in a similar inhibition of transactivation. To determine whether MDM2 interferes with the activity of the MyoD activation domain, we assayed the activity of either full-length MyoD fused to the DNA binding domain of GAL4 (GALMyoD) or the activation domain of MyoD fused to the DNA binding domain of GAL4 (GALN). The transcriptional activity of these constructs was assayed on the GAL-dependent reporter construct, GALCAT. As a control for MDM2 function, we assayed a fusion protein containing the activation domain of p53 fused to the DNA binding domain of GAL4, GALp53. These observations indicate that MDM2 does not interfere with the activity of the MyoD activation domain when fused to the DNA binding domain of GAL4. However, MDM2 deletion mutants, as indicated, were tested for inhibition of MyoD activity on the MCKCAT reporter construct. Inhibition of p53 activity on the p21CAT reporter by MDM2 was also assayed. * binding between MDM2 and pRB or Sp1 was determined previously (2).
dependent on Sp1 binding to Sp1 binding sites located in many muscle-specific genes (17), these observations suggested that MDM2 interferes with MyoD activity by interfering with Sp1 function.

To determine which domain of MDM2 was responsible for inhibition of MyoD, we assayed a series of MDM2 deletion mutants for the ability to interfere with MyoD-dependent transactivation using this assay. Again, as a control, we assayed inhibition of p53 activity by co-transfected MDM2. As reported previously (5), the N-terminal 220 amino acids of MDM2 are sufficient to inhibit p53-dependent transactivation (Fig. 2C). In contrast, co-transfection of MDM2 constructs containing the central acidic domain plus the first zinc-binding region was sufficient for inhibition of MyoD activity (Fig. 2C).

In addition, we have been unable to detect a direct interaction between MDM2 and MyoD, suggesting that inhibition of MyoD activity by MDM2 is indirect.2 However, because the region of MDM2 that inhibits MyoD binds to both Sp1 and pRb (2), these observations suggest that pRb and/or Sp1 were potential targets for MDM2 inhibition.

Forced Expression of Either pRb or Sp1 Can Restore Myogenesis—We previously found that cells with amplified MDM2 retain very little Sp1 transcriptional activity (2). Therefore, to test whether lower levels of Sp1 activity are responsible for the loss of MyoD function in cells with amplified MDM2, we assayed expression of 4RTKCAT in cells with amplified MDM2 transfected with an Sp1 expression vector. Fig. 3A shows that forced expression of Sp1 results in a significant increase in the activity of this reporter, suggesting that Sp1 activity may be limiting. This is in contrast to activation of this reporter by MyoD (see Fig. 1D).

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Fig. 3. Rescue of MyoD activity by Sp1 and pRb. A, synergistic activation of the 4RTKCAT reporter by Sp1 and pRb. NIH3T3 cells were transfected with the reporter construct p4RTKCAT, Sp1, and pRb as indicated. The amount of plasmid (µg) transfected is shown. Error bars represent S.E. B, forced expression of Sp1 or pRb rescues muscle cell differentiation in cells with amplified MDM2. A C2C12 microcell hybrid containing amplified MDM2 (see Fig. 1) was transfected with expression vectors encoding Sp1, pRb, or pRb deletion mutants containing the C or A+B domains (see panel C). Cells were incubated in differentiation-inducing media for 3 days and stained with an antibody against MHC. The frequency of differentiated cells was determined by scoring the number of nuclei in MHC-positive myotubes; a minimum of 2000 nuclei was scored under each condition. Error bars represent S.E. C, graphic representation of the pRb deletion mutants, with the amino acids, E2F, MyoD, and MDM2 binding domains indicated. D, forced expression of Sp1 or pRb induces premature differentiation. C2C12 cells were transfected with expression vectors encoding Sp1, pRb (WT), or the pRb deletion mutants C or A+B. An expression vector for green fluorescent protein (GFP) was co-transfected to control for transfection efficiency. Cells were maintained in growth media for 2 days and processed for Western blot analysis using MHC, troponin T, MyoD, and green fluorescent protein antibodies.
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MDM2. Fig. 3A also shows that forced expression of pRb results in stimulation of the transcriptional activity from this reporter construct, and that co-transfection of pRb and Sp1 results in "superactivation" of this reporter. These observations suggest that forced expression of either Sp1 or pRb can restore MyoD activity in cells with amplified MDM2. To test directly whether Sp1 and pRb can restore muscle differentiation in cells with amplified MDM2, we assayed the frequency of MHC-positive cells following transfection of the Sp1 and pRb expression vectors. Fig. 3B shows that transfection of either Sp1 or pRb results in a large increase in the number of MHC-positive cells. These observations indicate that forced expression of either Sp1 or pRb can restore MyoD activity and muscle cell differentiation in cells with amplified MDM2. To determine which domain of pRb is responsible for restoring differentiation in cells with amplified MDM2, we transfected these cells with expression vectors containing either the A+B pocket domain or the C-terminal MDM2-interacting domain (Fig. 3C). Fig. 3B shows that forced expression of the C domain results in more MHC-positive cells than forced expression of either Sp1 or full-length pRb. In contrast, forced expression of the A+B domain results in only a small increase in the frequency of MHC-positive cells. Taken together these observations indicate that forced expression of either Sp1 or pRb can restore MyoD and muscle cell differentiation in myoblast cells with amplified MDM2.

To determine whether Sp1 and pRb can modulate muscle cell differentiation in the absence of MDM2 amplification, we transfected parental C2C12 cells with expression vectors for Sp1, full-length pRb, and the pRb deletion mutants. Muscle differentiation was assayed in cells maintained in high serum media by Western blot analysis for the muscle-specific proteins Sp1, full-length pRb, and the pRb deletion mutants. Fig. 3B shows that transfection of the Sp1 and pRb expression vectors results in an increase in both MHC and troponin T expression, suggesting that Sp1 and pRb can promote premature differentiation of normal myoblast cells. In addition, transfection of the C-terminal MDM2-interacting domain of pRb also resulted in an increase in expression of these muscle markers. In contrast, forced expression of the A+B pocket of pRb had little or no affect on muscle differentiation in this assay, suggesting that the MyoD-interacting domain of pRb is not required for this activity. These observations indicate that forced expression of either Sp1 or pRb can induce premature differentiation and that the MDM2-interacting domain of pRb is both necessary and sufficient for this activity.

Regulation of E2F Activity by Sp1, pRb, and MDM2—Differentiating myoblasts fuse to form multinucleated myotubes and permanently withdraw from the cell cycle. This process requires the hypophosphorylated and activated form of pRb (19, 20). One important function of pRb is to bind and inhibit the S-phase-promoting transcription factor E2F (21). Furthermore, pRb can promote muscle cell differentiation by stimulating Sp1 activity (see above). Paradoxically, Sp1 has been implicated in the activation of muscle-specific genes by MyoD to high levels requires pRb (19, 28) or high levels of the pRb-related protein p107 (29). Loss of pRb function in muscle cells has two prominent phenotypes: 1) the level of activation of terminal differentiation markers is drastically reduced, and 2) new DNA synthesis can occur in the nuclei of the rare differentiated myotubes. During muscle differentiation, pRb expression increases (30–32) and assumes a hypophosphorylated and activated state (19, 20). Direct protein-protein interaction between pRb and MyoD has been proposed to account for the involvement of pRb during muscle cell differentiation (19). However, we show here that the C-terminal domain of pRb, which does not interact with MyoD, can also promote myogenic differentiation. In addition, pRb has been shown to modulate transcriptional activation as well as transcriptional repression. Transcriptional activation mediated by pRb occurs through a poorly understood mechanism that involves promoter elements called retinoblastoma control elements (RCEs), which are bound by Sp1 in vitro and are stimulated by Sp1 in vivo (33–35). Furthermore, co-expression of Sp1 and pRb results in "superactivation" of Sp1-mediated transcription (35). We recently found that MDM2 binds directly to Sp1 and inhibits Sp1 mediated transcription and that pRb can restore Sp1 activity by displacing Sp1 from MDM2 (2). In this report we show that pRb can facilitate MyoD function and muscle cell differentiation by restoring Sp1 activity in cells with amplified MDM2. In addition, we have shown that pRb results in the synergistic activation of this promoter. Furthermore, consistent with previous observations that pRb binds to MDM2 (12, 13) and that this interaction releases Sp1 from MDM2 (2), we find that pRb and MDM2 can be co-immunoprecipitated in cells transfected with the pRb expression vector (Fig. 4A). However, if the DHFR promoter is activated by E2F, pRb can now repress expression of this promoter (Fig. 4B). Furthermore, we find that E2F and pRb are associated under these conditions (Fig. 4B). To determine whether the DHFR stimulatory activity of pRb is associated with its C-terminal MDM2-interacting domain, we assayed expression of the DHFR promoter in cells co-transfected with the different pRb deletion mutants. Fig. 4C shows that co-transfection of full-length pRb or the A+B domain of pRb resulted in inhibition of E2F-mediated activation of the DHFR promoter. In contrast, co-transfection of the C-terminal MDM2-interacting domain of pRb results in stimulation of the DHFR promoter. These observations suggest that in the absence of co-transfected E2F, pRb induces expression from the DHFR promoter through stimulation of Sp1 activity, and that in the presence of co-transfected E2F, pRb represses expression from this promoter. Because E2F and Sp1 have been shown to interact directly and synergistically activate the DHFR promoter, and because pRb interacts with E2F and inhibits E2F-mediated transactivation, we next tested whether the E2F-pRb interaction could interfere with the E2F-Sp1 interaction. For this experiment, we used an in vitro pull-down assay using in vitro translated E2F1 and GST-Sp1. Fig. 4D shows that E2F1 interacts with GST-Sp1, and not with GST alone, and that titration of increasing amounts of bacterially expressed and purified pRb interferes with the E2F1-Sp1 interaction. This observation suggests that pRb can inhibit E2F-dependent transactivation by interfering with the E2F-Sp1 interaction and preventing synergistic transactivation.

DISCUSSION

Muscle cell differentiation is accompanied by terminal cell cycle withdrawal. This process is controlled by regulatory interactions between MyoD family members and various cell cycle regulatory proteins (27). One important interaction involves the tumor suppressor protein pRb. Transactivation of muscle-specific genes by MyoD to high levels requires pRb (19, 28) or high levels of the pRb-related protein p107 (29). Loss of pRb function in muscle cells has two prominent phenotypes: 1) the level of activation of terminal differentiation markers is drastically reduced, and 2) new DNA synthesis can occur in the nuclei of the rare differentiated myotubes. During muscle differentiation, pRb expression increases (30–32) and assumes a hypophosphorylated and activated state (19, 20). Direct protein-protein interaction between pRb and MyoD has been proposed to account for the involvement of pRb during muscle cell differentiation (19). However, we show here that the C-terminal domain of pRb, which does not interact with MyoD, can also promote myogenic differentiation. In addition, pRb has been shown to modulate transcriptional activation as well as transcriptional repression. Transcriptional activation mediated by pRb occurs through a poorly understood mechanism that involves promoter elements called retinoblastoma control elements (RCEs), which are bound by Sp1 in vitro and are stimulated by Sp1 in vivo (33–35). Furthermore, co-expression of Sp1 and pRb results in "superactivation" of Sp1-mediated transcription (35). We recently found that MDM2 binds directly to Sp1 and inhibits Sp1 mediated transcription and that pRb can restore Sp1 activity by displacing Sp1 from MDM2 (2). In this report we show that pRb can facilitate MyoD function and muscle cell differentiation by restoring Sp1 activity in cells with amplified MDM2. In addition, we have shown that pRb...
FIG. 4. Regulation of the DHFR promoter by Sp1, pRb, and E2F. A, activation of the DHFR promoter by Sp1 and pRb. NIH3T3 cells were transfected with the DHFRLUC reporter construct and increasing amounts of Sp1 and pRb. Shaded bars and black bars represent reporter activity from duplicate samples. A representative experiment is shown, and similar results were obtained in replicate experiments. Cell extracts were assayed for luciferase activity and for expression of Sp1 and pRb by Western blot analysis (IB-pRB). B, inhibition of the DHFR promoter by pRb in the presence of E2F. NIH3T3 cells were co-transfected with the DHFRLUC reporter construct, an E2F expression vector, and increasing amounts of pRb expression vector. Shaded bars and black bars represent reporter activity from duplicate samples. A representative experiment is shown, and similar results were obtained in replicate experiments. Expression of E2F and pRb was analyzed by Western blot analysis. The interaction between E2F and pRb was assayed by co-immunoprecipitation with an antibody against E2F (IP-E2F). C, stimulation of E2F activity by the C terminus of pRb. NIH3T3 cells were transfected with the DHFRLUC reporter construct, E2F, and expression vectors encoding full-length pRb, the A+B domain, or the C-terminal domain of pRb. Shaded bars and black bars represent reporter activity from duplicate samples. A representative experiment is shown, and similar results were obtained in replicate experiments. Expression of pRb, E2F, Sp1, pRb, the pRb mutants (FLAG), and γ-tubulin were assayed by Western blot analysis. D, pRb interferes with the E2F-Sp1 interaction. In vitro translated and radiolabeled E2F was subjected to in vitro pull-down using GST-Sp1. The E2F-Sp1 complex was incubated with increasing amounts (25, 50, and 100 ng) of purified pRb.
Our results support a model in which pRb and MDM2 regulate muscle cell differentiation by modulating Sp1 activity. pRb facilitates MyoD function and muscle cell differentiation by competing with MDM2 for Sp1 binding. Sp1 transcriptional activity is regulated by physical interaction with MDM2, and this interaction inhibits DNA binding to muscle-specific promoters. pRb can reverse the inhibitory effects of MDM2 by physically interacting with the MDM2-Sp1 complex and releasing free Sp1, thus restoring DNA binding and transcriptional activation to muscle-specific promoters. In the presence of amplified MDM2, pRb never reaches high enough levels to release Sp1 from the MDM2-Sp1 complex. MDM2 stimulates E2F activity on S-phase-specific promoters by competing with pRb for E2F binding and by recruiting Sp1. In the presence of amplified MDM2, pRb becomes limiting. The S-phase-specific promoters are bound by E2F, MDM2, and Sp1 even under differentiation promoting conditions.
can induce premature differentiation of normal proliferating myoblasts maintained in growth media. Our results support a model in which pRb and MDM2 regulate muscle cell differentiation by modulating Sp1 activity (Fig. 5). Sp1 transcriptional activity is regulated by physical interaction with MDM2, and this interaction inhibits DNA binding to muscle-specific promoters. Furthermore, pRb can reverse the inhibitory effects of MDM2 by physically interacting with the MDM2-Sp1 complex and releasing free Sp1, thus restoring DNA binding and transcriptional activation. Amplification and over-expression of MDM2 occur in ~30% of human sarcomas (3). Our model predicts that in cells with amplified MDM2, pRb becomes limiting, and as a consequence, transcription from genes dependent on Sp1 activity is reduced. In addition, another important role for pRb during cellular differentiation is to bind and inhibit E2F activity, thus preventing entry into S-phase (36). We have found that pRb interferes with the E2F-Sp1 interaction, suggesting that an additional role for pRb in the regulation of cell cycle progression is to interfere with the synergistic activation of S-phase-specific promoters by E2F and Sp1. Because Sp1 has been implicated in the expression of genes involved in both terminal differentiation, e.g. human cardiac actin (16, 17), as well as S-phase, e.g. DHFR (22) and thymidine kinase (23), our model suggests that pRb and MDM2 control the decision between cell cycle withdrawal during differentiation and entry into S-phase during proliferation by modulating the availability of Sp1.

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