

Retinoblastoma Protein and MyoD Function Together to Effect the Repression of Fra-1 and in Turn Cyclin D1 during Terminal Cell Cycle Arrest Associated with Myogenesis*

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Background: Repression of cyclin D1 is required for terminal cell cycle arrest associated with myogenesis.

Results: pRb and MyoD function together to block induction of Fra-1 and in turn cyclin D1.

Conclusion: MyoD and pRb cooperate to effect transcriptional repression during myogenesis.

Significance: The findings reveal a link between MyoD, pRb, Fra-1, and cyclin D1, the latter three of which are often deregulated in cancer.

The acquisition of skeletal muscle-specific function and terminal cell cycle arrest represent two important features of the myogenic differentiation program. These cellular processes are distinct and can be separated genetically. The lineage-specific transcription factor MyoD and the retinoblastoma protein pRb participate in both of these cellular events. Whether and how MyoD and pRb work together to effect terminal cell cycle arrest is uncertain. To address this question, we focused on cyclin D1, whose stable repression is required for terminal cell cycle arrest and execution of myogenesis. MyoD and pRb are both required for the repression of cyclin D1; their actions, however, were found not to be direct. Rather, they operate to regulate the immediate early gene *Fra-1*, a critical player in mitogen-dependent induction of cyclin D1. Two conserved MyoD-binding sites were identified in an intronic enhancer of *Fra-1* and shown to be required for the stable repression of *Fra-1* and, in turn, cyclin D1. Localization of MyoD alone to the intronic enhancer of *Fra-1* in the absence of pRb was not sufficient to elicit a block to *Fra-1* induction; pRb was also recruited to the intronic enhancer in a MyoD-dependent manner. These observations suggest that MyoD and pRb work together cooperatively at the level of the intronic enhancer of *Fra-1* during terminal cell cycle arrest. This work reveals a previously unappreciated link between a lineage-specific transcription factor, a tumor suppressor, and a proto-oncogene in the control of an important facet of myogenic differentiation.

Myogenic differentiation involves the execution of various processes including the acquisition of skeletal muscle-specific function and terminal cell cycle arrest. The principal effectors

of these processes are the myogenic basic helix-loop-helix transcription factors: MyoD (MyoD1), Myf5, myogenin (Myog), and MRF4 (Myf6), that affect transcription following binding to specific DNA sequences (CANNTG) termed an E box. Indeed, ectopic expression of a single myogenic transcription factor, MyoD, is sufficient to initiate the myogenic program (1, 2).

When wild-type myoblasts are grown under low mitogen conditions that induce myogenesis, they withdraw from the cell cycle and begin to differentiate. If, following completion of the program, the differentiated myoblasts are restimulated in the presence of high mitogen containing serum, they fail to re-enter the cell cycle—this being a functional definition of terminal cell cycle arrest. How terminal cell arrest is established and maintained is poorly understood.

The retinoblastoma protein, pRb (Rb1), like MyoD, has been shown to play a significant role in several aspects of myogenesis, and this is supported by both *in vivo* and *in vitro* data. Skeletal muscle deficient for *Rb* shows reduced fiber density, a lack of expression of late markers of differentiation (e.g. *muscle creatine kinase*, *MCK* (*Ckmm*)), and ectopic proliferation (3–6). The cell culture correlates of these observations are manifest in *Rb*-deficient myoblasts by the inability of MyoD to induce *MCK* expression and the failure to undergo terminal cell cycle arrest (7, 8). The role of pRb in mediating these two processes, however, is distinct, and the same is true of MyoD.

At the level of pRb, its abilities to stimulate skeletal muscle differentiation and bring about terminal cell cycle arrest are genetically separable, as revealed by *in vivo* and *in vitro* analyses (6, 9, 10), and both of these processes do not require the well characterized E2F-dependent G₁ phase cell cycle control function of pRb (9, 11–14). The role of pRb in MyoD-mediated stimulation of *MCK* expression has been traced to the transactivation function of MEF2C (6, 15), a member of the MEF2 family of transcription factors that cooperates with MyoD to effect muscle-specific gene activation. With respect to MyoD, its requirements for effecting differentiation and terminal cell cycle arrest are different (16, 17). Notwithstanding these insights, whether MyoD and pRb actually collaborate to effect

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pRb and MyoD Function during Terminal Cell Cycle Arrest

terminal cell cycle arrest and, if so, the nature of this collaboration are not known.

To gain a greater appreciation for the myogenic program, several groups have defined the changes in gene expression during differentiation on a global scale (18–23). A number of salient observations from these studies are pertinent to the work presented here. In the canonical view of MyoD function, it is portrayed as a transcriptional activator; however, a role for MyoD in directly repressing transcription during myogenesis was revealed (18, 22, 23). In terms of differentiation-associated terminal cell cycle arrest, one study noted that surprisingly few cell cycle regulatory genes were affected by MyoD, leading the authors to propose that MyoD-mediated control of the cell cycle may be achieved indirectly through the regulation of other transcription factors (22). A role for MyoD in inhibiting transcription in a cycling, undifferentiated state has been described (24), but this mechanism is unlikely to operate during myogenic differentiation and terminal cell cycle arrest. Despite these efforts, how MyoD blocks transcription and the relevance of this function to myogenesis remain to be determined.

Although several mechanisms have been proposed for withdrawal from the cell cycle during myogenesis (25–30), a significant event that takes place during terminal cell cycle arrest is the stable repression of cyclin D1 (*Ccnd1*). The importance of the block to cyclin D1 induction upon restimulation of differentiated myoblasts is indicated by the observation that ectopic expression of cyclin D1 can drive differentiated myoblasts into the S phase by effecting the phosphorylation and inactivation of pRb (31). Further, cyclin D1 has been shown to inhibit the transcriptional activity of MyoD, myogenin, and MEF2C, thereby hindering their ability to execute the myogenic differentiation program (32–35). These observations suggest that the repressed state of cyclin D1 is essential to the maintenance of terminal cell cycle arrest and the faithful execution of the myogenic differentiation program. However, how the repressed state of cyclin D1 is achieved is uncertain.

Here we have explored the role of MyoD and pRb in terminal cell cycle arrest during the myogenic program of differentiation. We report that MyoD and pRb work together to effect terminal cell cycle arrest. Their cooperative actions inhibit the induction of cyclin D1 during mitogenic restimulation of differentiated myoblasts; however, the actions of MyoD and pRb are not direct. Rather, we find that MyoD and pRb directly block the induction of *Fra-1* (*Fosl1*), an immediate early gene product and an upstream participant effecting the expression of cyclin D1. We identified conserved sequences in the *Fra-1* gene that are responsible for the recruitment of MyoD, an event that leads to the localization of pRb to the *Fra-1* gene; both MyoD and pRb are required for the inhibition of *Fra-1* and cyclin D1 induction in differentiated myoblasts. Collectively, these observations reveal a previously unappreciated role for MyoD and pRb in mediating a block to transcription during a salient facet of myogenesis: terminal cell cycle arrest.

EXPERIMENTAL PROCEDURES

Cell Culture—Immortalized 3T3 derivatives of *Rb*^{+/+} and *Rb*^{-/-} mouse embryo fibroblasts have been described (6, 10, 36) and were cultured in DMEM supplemented with 10% FBS.

The C2C12 murine myoblast line (37) was cultured in DMEM supplemented with 20% FBS. *Rb*^{+/+} and *Rb*^{-/-} myoblasts (3T3 derivatives infected with retrovirus encoding MyoD) and C2C12 cells were differentiated in DMEM containing 2% horse serum for 6 days with a change in media every 2 days. The cells were restimulated with the addition of DMEM containing 20% FBS. C2C12 cells were rendered quiescent by culturing in 0.1% FBS for 3 days.

Plasmids, Infections, and Antibodies—Retroviral vectors encoding MyoD (a gift from Andrew Lassar) (8) and p16 (38) were used to perform infections as described (10, 39). Murine *Fra-1* (a gift from Tim Rothstein) was subcloned into pShuttle-CMV (a gift from Bert Vogelstein), and recombinant adenovirus was prepared as described (40). Ad-GFP was a gift from Kornelia Polyak. Infections were performed at a multiplicity of infection of 100. Antibody against cyclin D1 (Ab-3) was from Lab Vision (Thermo Scientific); antibodies against MyoD (M318, sc-760 and C-20, sc-304), *Fra-1* (N-17, sc-183), *Fra-2* (Q-20, sc-604), c-Fos (4, sc-52), and c-Jun (N, sc-45) were from Santa Cruz Biotechnology; antibody against pRb (G3-245) was from BD Pharmingen (BD Biosciences); antibody against p16 was a gift from James Decaprio; and antibodies against β -actin (catalog number A5441) and tubulin (catalog number T5168) were from Sigma.

***Fra-1* Promoter/Enhancer Luciferase Reporter**—A genomic fragment of the murine *Fra-1* (*Fosl1*) gene spanning 5'-upstream sequences, exon 1, intron 1, and part of exon 2 (a gift from Erwin Wagner) (41) was used to create a *Fra-1* promoter/enhancer reporter. Sequences running from -930 to +2491 (41) relative to the transcriptional start site were subcloned such that sequences in exon 2 were in frame with *luciferase* in the pGL-3 Basic vector (Promega). Mutant *Fra-1* promoter/enhancer reporters were generated by site-directed mutagenesis (Stratagene) and confirmed by sequencing. The following mutations were introduced: E box 1, CAGGTG > TCAGGC; E box 2, CACGTG > TCAGGC; and E box 3, CACCTG > TCAGGC. To generate stable lines of each promoter/enhancer reporter construct, the *Rb*^{+/+} and *Rb*^{-/-} 3T3 fibroblasts were co-transfected with *Fra-1* promoter/enhancer reporter and pBabe-puro plasmid (5:1 ratio) using Superfect transfection reagent (Qiagen). Cells were selected under 3 μ g/ml puromycin (Calbiochem) to generate pooled populations of *Fra-1* promoter/enhancer reporter lines.

Transcriptional Activation Assays—Stable lines harboring an integrated *Fra-1* promoter/enhancer reporter and its mutant derivatives were infected either with retrovirus encoding MyoD or “empty” vector. Forty-eight hours later, the cells were induced to differentiate by culturing in medium containing 2% horse serum for 3 days. The cells were restimulated with media containing 20% FBS, normalized for protein content, and luciferase activity was measured as described (42). Statistical analyses were performed using Student's *t* test.

Chromatin Immunoprecipitation—ChIP was carried out essentially as described (43). The cells were cross-linked with 1% formaldehyde solution at 37 °C for 10 min followed by two quick washes with cold PBS on ice. The cells were scraped into cold PBS and either stored at -80 °C or used immediately for ChIP. Briefly, the cells were lysed with ChIP lysis buffer (50 mM

Tris-HCl, pH 8.0, 1% SDS, 5 mM EDTA, plus protease inhibitors; Roche) on ice for 10–20 min and sonicated five times at 12% amplitude for 15 s (Fisher Scientific model 500 Sonic Dismembrator). The majority of DNA fragments were below 500 bp. Samples were subjected to centrifugation at $16,000 \times g$ for 20 min, and 100 μ l of supernatant was mixed with 900 μ l of ChIP dilution buffer (20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, plus protease inhibitors). Lysates were incubated overnight at 4 °C with specific antibodies to MyoD (M318, sc-760; Santa Cruz Biotechnology), pRb (clone G3-245; BD Biosciences), RNA polymerase II (C-21, sc-900; Santa Cruz Biotechnology), or appropriate mouse and rabbit IgG as controls. Immune complexes were incubated with protein A or protein G-Sepharose beads (Santa Cruz Biotechnology) for 1 h and then washed with ChIP wash buffers TSE I (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl), TSE II (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), and buffer III (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA), followed by two washes with TE (pH 8.1) for 10 min each at 4 °C. Samples were eluted from the beads with 100 μ l of 1% SDS, 0.1 M NaHCO₃ for 20 min and incubated overnight at 65 °C to reverse cross-linking. DNA was purified using Qiagen PCR purification kit and eluted in 100 μ l of elution buffer (Qiagen). PCR was carried out with specific primers using Taq polymerase kit (Qiagen). Primer sequences are as follows: intron 1 of *Fra-1* (Fra-1 int 1) +, 5'-AGGTTGCAGGTGTCCATTTCCTGT-3'; intron 1 of *Fra-1* (Fra-1 int 1) -, 5'-TGCTTAGGCAAGTCTGGACAGCTA-3'; control 3 kb downstream (Fra-1 3 kb) +, 5'-GCACTGAATGCACAAGGTGCTCAT-3'; control 3 kb downstream (Fra-1 3 kb) -, 5'-ACGGTGGCTCACAACCACCTATAA-3'; transcriptional start site in *Fra-1* (Fra-1 TSS) +, 5'-TGTGTTGGGAACCTGGCTAGTCT-3'; transcriptional start site in *Fra-1* (Fra-1 TSS) -, 5'-AAGTTCTTGGGCTGAACCACTTGC-3'; MCK enhancer (MCK) +, 5'-GTCTAGGCTGCCCATGTAAGG-3' (44); and MCK enhancer (MCK) -, 5'-CAGGCCAGGAAGGATACAG-3' (44).

RESULTS

Specific Block to *Fra-1* Induction during Terminal Cell Cycle Arrest—Given that members of the AP-1 family of transcription factors are implicated in the mitogenic induction of cyclin D1 (45), we hypothesized that a block to one or more AP-1 members might be responsible for the corresponding inhibition of cyclin D1 induction. C2C12 myoblasts were cultured under conditions known to induce a reversible quiescent state or an irreversible differentiated state and then restimulated by the addition of 20% fetal bovine serum. Next, the induction of the protein products encoded by several immediate early genes (*c-Fos*, *c-Jun*, *Fra-1*, *Fra-2*, *JunB*, and *JunD*) and cyclin D1 was monitored. The expression of all of these proteins was induced when quiescent myoblasts were restimulated (Fig. 1; data not shown). A different scenario emerged when differentiated (as opposed to quiescent) C2C12 cells were restimulated. Here, many of the proteins encoded by immediate early genes were induced (Fig. 1; data not shown; see also Fig. 2), consistent with previous findings (46). This occurred with the striking excep-

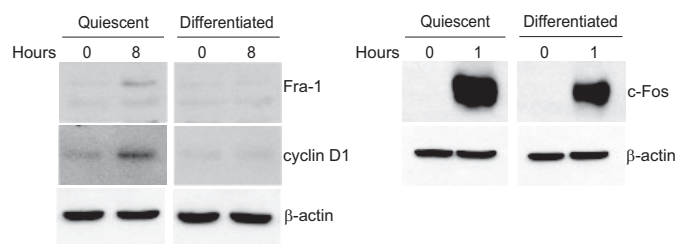


FIGURE 1. *Fra-1* and cyclin D1 are induced upon restimulation of quiescent but not differentiated myoblasts. C2C12 cells were rendered quiescent by culturing in 0.1% FBS for 3 days or differentiated by culturing in 2% horse serum for 6 days. Subsequently, the cells were restimulated with 20% FBS for 8 h (left panel) or 1 h (right panel). The lysates were prepared and subjected to immunoblotting with antibodies to the indicated proteins. β -Actin immunoblots were used to assess protein loading. The results are representative of at least three independent experiments.

tion of *Fra-1*, whose expression was not induced specifically upon restimulation of differentiated myoblasts (Fig. 1). In a similar fashion, cyclin D1 was not induced in differentiated myoblasts upon restimulation; cyclin D1 was induced upon restimulation of quiescent myoblasts (Fig. 1). These observations suggest that *Fra-1* is unique among immediate early gene products in its inability to be induced upon serum stimulation of differentiated myoblasts, an event that is coincident with a similar lack of induction of cyclin D1.

MyoD and pRb Cooperate to Effect Terminal Cell Cycle Arrest—Next, we determined whether both MyoD and pRb might participate in the block to induction of *Fra-1* and cyclin D1. *Rb*^{+/+} and *Rb*^{-/-} fibroblasts were infected with a retrovirus encoding MyoD or empty vector. Following infection, cells were cultured under conditions known to induce myogenic differentiation. Subsequently, the expression of *Fra-1* and cyclin D1 was monitored upon restimulation of the cells. In *Rb*-deficient myoblasts *Fra-1* was induced (Fig. 2A), and correspondingly, cyclin D1 expression also increased, consistent with previous observations (7). Similar observations were made in restimulated *Rb*-positive cells infected with empty vector (Fig. 2A). By contrast, in the presence of both MyoD and pRb, *Fra-1* and cyclin D1 were not induced (Fig. 2A). When assayed at later times in *Rb*^{+/+} myoblasts, induction of *Fra-1* and cyclin D1 was still not detected, thus ruling out the possibility that their expression was delayed (data not shown). The effect of MyoD and pRb on *Fra-1* induction was specific, because proteins encoded by other immediate early genes were induced regardless of the presence of MyoD or pRb (Fig. 2, A and B). A consistent decline in *c-Fos* was noted in the presence of MyoD, but independent of pRb, in agreement with previous findings (26). Further, ectopic expression of the cdk4 and cdk6 inhibitor p16 (Cdkn2a) did not prevent the induction of either *Fra-1* or cyclin D1 (Fig. 2C), reinforcing the notion that a G₁ arrest mediated by pRb-E2F complexes is distinct from the irreversible arrest brought about by pRb during myogenic differentiation. Together, these observations suggest that pRb and MyoD establish and perhaps maintain terminal cell cycle arrest, in part, by preventing the induction of *Fra-1* and cyclin D1.

Fra-1 Acts Upstream of Cyclin D1—The results presented above, together with the observation that AP-1 family members participate in the serum induction of cyclin D1, suggest that the block to *Fra-1* induction is causally related to the corresponding

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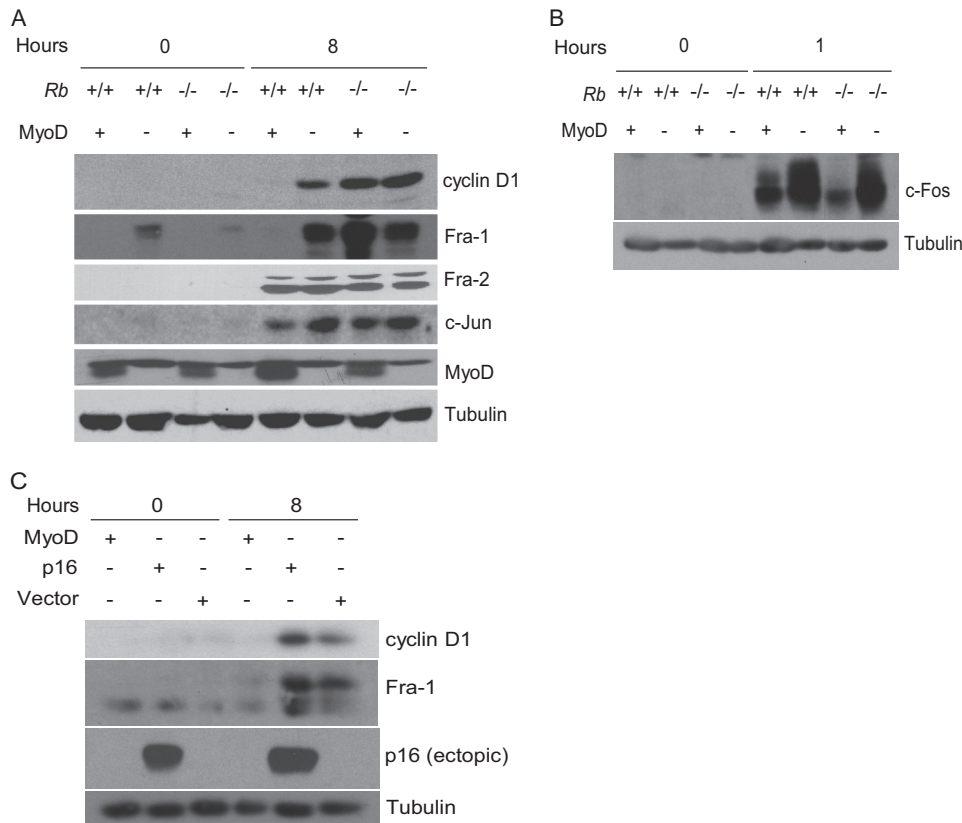


FIGURE 2. Regulation of Fra-1 and cyclin D1 by MyoD and pRb. *A*, block to Fra-1 and cyclin D1 induction is MyoD- and pRb-dependent. $Rb^{+/+}$ and $Rb^{-/-}$ fibroblasts (indicated) were infected with virus directing the expression of MyoD or empty vector and cultured under conditions to induce differentiation (2% horse serum) for 3 days (0-h time point). Subsequently, cells were restimulated for 8 h with 20% FBS. Lysates were prepared and subjected to immunoblotting with antibodies to the indicated proteins. Tubulin immunoblots were used to assess protein loading. The results are representative of at least three independent experiments. *B*, as in *A*, except c-Fos was analyzed at 1 h after restimulation. *C*, p16 induces a G_1 arrest independent of down-regulation of Fra-1. $Rb^{+/+}$ fibroblasts were infected with MyoD- or p16-encoding virus or empty vector and cultured under conditions to induce myogenic differentiation for 3 days (0-h time point). Subsequently, cells were restimulated for 8 h with 20% FBS. Expression of the indicated proteins was analyzed by immunoblotting. Lysates were prepared and subjected to immunoblotting with antibodies to the indicated proteins. Tubulin immunoblots were used to assess protein loading. The results are representative of at least three independent experiments.

block to cyclin D1 induction. Consistent with this possibility and in keeping with previous observations (47, 48), expression of Fra-1 preceded that of cyclin D1 following restimulation of quiescent $Rb^{+/+}$ and $Rb^{-/-}$ fibroblasts, as well as C2C12 cells (Fig. 3A). That Fra-1 and cyclin D1 were induced earlier in $Rb^{-/-}$ compared with $Rb^{+/+}$ fibroblasts is consistent with the observation that the former traverse G_1 faster (49).

To determine whether Fra-1 operates upstream of and in series with cyclin D1, we asked whether ectopic expression of Fra-1 in differentiated myoblasts can induce the expression of cyclin D1 upon serum restimulation. To this end, recombinant adenoviruses were used to ectopically express Fra-1 or GFP as a control in differentiated C2C12 cells. Upon serum restimulation, cyclin D1 was induced in cells expressing Fra-1, but not GFP (Fig. 3B), suggesting that the block to Fra-1 induction is responsible for the lack of cyclin D1 induction. Similar observations were made with wild-type Rb myoblasts (Fig. 3C). As anticipated, cyclin D1 was not induced under differentiation conditions but required serum restimulation. This follows from the fact that Fra-1 on its own cannot mediate transcriptional induction, because it lacks a transactivation domain and must heterodimerize with mitogen-induced Jun family members to affect transcription (50–52). Together with the findings noted above, these observations suggest that the *cyclin D1* gene is not

rendered refractory to induction following myogenic differentiation. Rather, the actions of MyoD and pRb in effecting the block to cyclin D1 induction are indirect and mediated at the level of the *Fra-1* gene.

MyoD Binds to Sites Located in Intron 1 of Fra-1—To determine how MyoD and pRb impose a block to Fra-1 induction, we considered a region previously identified as an intronic enhancer in the *Fra-1* gene responsible for its basal and serum-inducible expression. Specifically, three closely spaced AP-1 (and AP-1 like) sites located in the first intron of the *Fra-1* gene have been shown to play a significant role in the induction of the *Fra-1* during mitogen-induced cell cycle re-entry from a quiescent state (52, 53). We searched for other possible conserved regulatory elements within the first 2000 bp of the first intron in the *Fra-1* gene comparing murine and human sequences using Bayes block aligner (54) (murine and human *Fra-1* intron 1 are 2105 and 3234 bps in length, respectively). This analysis revealed that the largest region displaying the highest degree of conservation contained the 5'-most AP-1 element (Fig. 4A); the other two AP-1 elements were located downstream in the adjacent conserved region. Of these two conserved regions identified in intron 1 of *Fra-1*, in the larger, 5'-most one, we noted two potential MyoD-binding sites (E boxes, CANNTG) juxtaposed to the

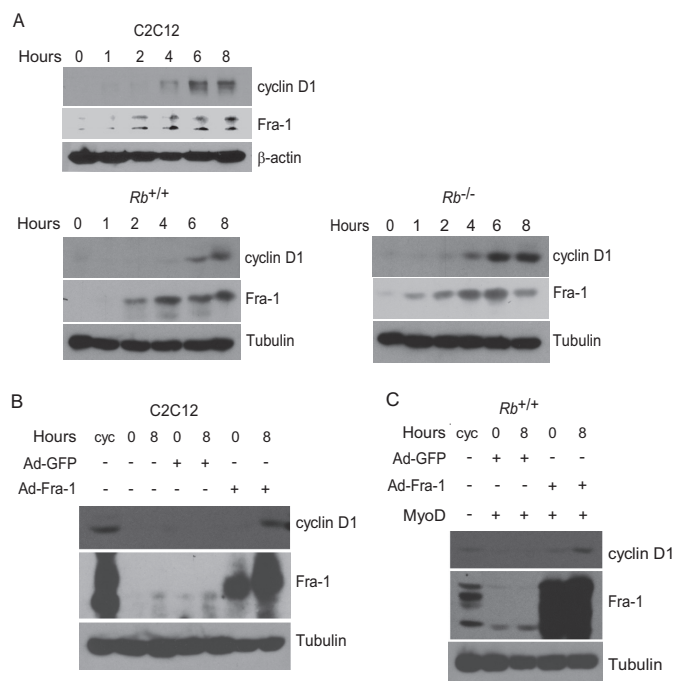


FIGURE 3. Fra-1 participates in the induction of cyclin D1. *A*, kinetics of Fra-1 and cyclin D1 induction. C2C12 cells (*upper panel*) were rendered quiescent by culturing in 0.1% FBS for 3 days and then restimulated by the addition of 20% FBS. Additionally, *Rb*^{+/+} (*lower left panel*) and *Rb*^{-/-} (*lower right panel*) fibroblasts were cultured under differentiation conditions for 3 days (which in the absence of MyoD renders these cells quiescent) and then restimulated by the addition of 20% FBS. At the indicated times, lysates were prepared and subjected to immunoblotting with antibodies to the indicated proteins. The results are representative of at least three independent experiments. *B*, Fra-1 operates upstream of cyclin D1 to induce its expression. C2C12 cells were cultured under conditions to induce their differentiation and then infected with adenovirus directing the expression of Fra-1 or GFP (0-h time point). Subsequently, cells were restimulated for 8 h with 20% FBS. A cycling (*cyc*) population of C2C12 cells was included for comparison. Expression of the indicated proteins at the indicated times was analyzed by immunoblotting. Tubulin immunoblots were used to assess protein loading. The results are representative of at least three independent experiments. *C*, as in *B* except *Rb*^{+/+} myoblasts were used. The results are representative of at least three independent experiments.

intronic enhancer of *Fra-1* (Fig. 4*A*), and the sequence of each of these two E box elements is identical among murine, rat, human, and chimp species.

MyoD-Binding Sites in Fra-1 Are Required to Block Its Induction—To evaluate the possible relevance of the conserved intronic E boxes, we asked whether MyoD localized to sequences in the first intron of the *Fra-1* gene using ChIP assays. C2C12 cells were differentiated, and soluble chromatin was prepared from formaldehyde-treated cells. Antibody specific for MyoD was then used to precipitate genomic DNA fragments bound to MyoD. This DNA was analyzed by PCR using a primer pair spanning the two E boxes identified in intron 1 of the *Fra-1* gene. A primer pair located 3 kb downstream of the E boxes was used as a negative control, and a primer pair to sequences in the *muscle creatine kinase* (*MCK*) enhancer, a known target of MyoD, was used as a positive control. These analyses revealed that MyoD was specifically bound to sequences in the first intron of the *Fra-1* gene in differentiated, but not cycling, myoblasts (Fig. 4*B*). Similar observations were made with *Rb*-positive and -negative myoblasts (Fig. 4, *C* and *D*). Results were confirmed with two different antibodies to

MyoD, and specificity was assessed with a lack of a signal in identically treated *Rb*^{+/+} and *Rb*^{-/-} fibroblasts (*i.e.* in the absence of MyoD) (Fig. 4, *C* and *D*; data not shown). Consistent with the presence of two conserved E boxes in intron 1 of the *Fra-1* gene, these results suggest that MyoD occupies these sites during differentiation and does so independent of *Rb* status.

To assess the functional significance of binding of MyoD to the conserved E box elements in the first intron of *Fra-1*, we generated a *Fra-1* promoter/enhancer reporter. Sequences from a murine genomic fragment extending from upstream of the transcriptional start sites through exon 1, intron 1, and part of exon 2 were cloned upstream of *luciferase* (Fig. 5*A*). In addition to this wild-type promoter/enhancer reporter, three mutants were generated in which sequences of the two conserved E boxes and one nonconserved E box were altered in such a way that they would be predicted to disrupt MyoD binding. The wild-type promoter/enhancer reporter was stably integrated into *Rb*^{+/+} and *Rb*^{-/-} fibroblasts and the mutant promoter/enhancer reporters into *Rb*^{+/+} fibroblasts. Subsequently, cells were infected with a retrovirus encoding MyoD or empty vector, differentiated, and then restimulated. As anticipated, under these conditions, the wild-type promoter/enhancer reporter was not induced following restimulation in an pRb- and MyoD-dependent manner (Fig. 5, *B* and *C*). By contrast, mutation of either or both of the conserved E boxes resulted in induction of the promoter/enhancer reporter regardless of whether MyoD was present (Fig. 5, *D–F*); the promoter/enhancer reporter bearing a mutation in the nonconserved E box behaved like the wild-type reporter (Fig. 5*G*). Together with our observation suggesting that MyoD can be localized to sequences in intron 1 of the *Fra-1* gene (Fig. 4), these findings provide functional evidence suggesting that MyoD binding to the conserved intronic E-boxes participates in imposing a block to *Fra-1* induction following restimulation of differentiated myoblasts. Further, MyoD localization to intron 1 of *Fra-1* alone is not sufficient to inhibit its induction; pRb must also be present, consistent with the results presented in Fig. 2*A*.

pRb Is Recruited to Intron 1 of Fra-1 in a MyoD-dependent Manner—The results presented above suggest that inhibition of Fra-1 induction following restimulation of differentiated myoblasts involves the actions of MyoD at the intronic enhancer. Further, pRb, in addition to MyoD, is required to impose the block to Fra-1 expression, leaving open the question as to what the role of pRb is in this process. Given the well documented role of pRb in repressing transcription (55) and the reported interaction between MyoD and pRb (25), we entertained the possibility that pRb is also recruited to the intronic enhancer of *Fra-1*. By ChIP analysis, we found that pRb was localized to the MyoD-binding sites within the intronic enhancer of *Fra-1* in differentiated, but not cycling, C2C12 cells (Fig. 6*A*), suggesting that MyoD and pRb act together at the intronic enhancer of *Fra-1*.

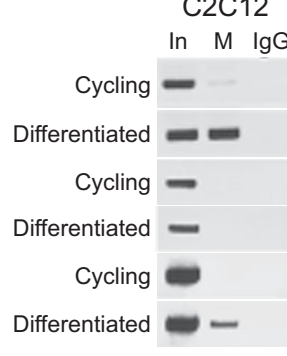
Next, we asked whether recruitment of pRb to the *Fra-1* gene was dependent of MyoD. *Rb*^{+/+} fibroblasts were transduced with a MyoD-encoding retrovirus or empty vector. We found that under differentiation conditions, pRb was localized to the intronic enhancer of *Fra-1*, but only in the presence of MyoD

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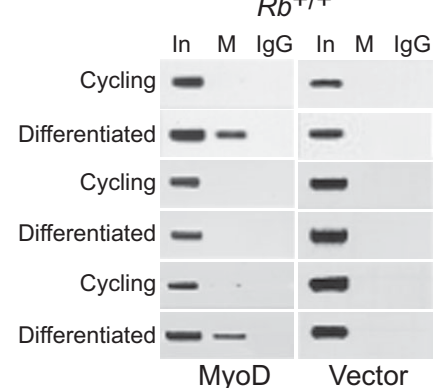
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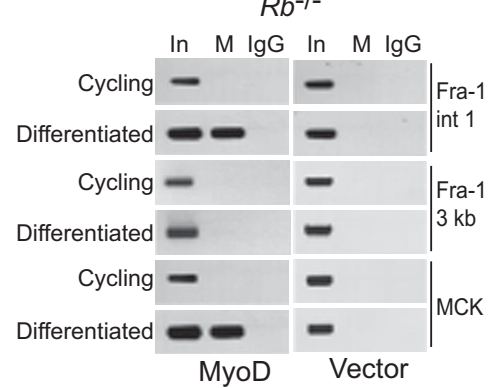


FIGURE 4. **MyoD is localized to E boxes located in the intronic enhancer of *Fra-1* during differentiation.** **A**, first 2000 bp of murine and human intron 1 of *Fra-1* were analyzed with Bayes block aligner (top). Probability of conservation: 0.8–0.999 (red), 0.6–0.799 (blue), 0.4–0.599 (yellow), 0.2–0.399 (green), and 0–0.199 (black). The largest region showing the highest degree of conservation is shown at the bottom, and E boxes and AP-1 sites are underlined (coordinates are defined by setting the first nucleotide of intron 1 to 1). **B**, MyoD localization to intronic E boxes of *Fra-1* during differentiation. ChIP analysis of cycling and differentiated C2C12 cells was performed with antibody to MyoD (M) or IgG as control. For the precipitated DNA, sequences spanning the E boxes in intron 1 of *Fra-1* (Fra-1 int 1), ~3 kb downstream of the *Fra-1* intronic E boxes (Fra-1 3 kb), and the *MCK* enhancer (MCK) were analyzed by PCR. One-tenth of the lysates used for ChIP were also subjected to PCR (input, In). The results are representative of at least three independent experiments. **C**, as in **B**, except *Rb*-positive fibroblasts were infected with MyoD-encoding virus (left panel) or empty vector (right panel). The results are representative of at least three independent experiments. **D**, as in **C**, except *Rb*-deficient fibroblasts were employed. The results are representative of at least three independent experiments.

(Fig. 6B). Localization of pRb was not observed in cycling cells either in the presence or absence of MyoD (Fig. 6B). The specificity of the ChIP for pRb was confirmed by performing similar experiments in *Rb*^{-/-} myoblasts and fibroblasts (Fig. 6C). These observations are consistent with a model in which MyoD recruits pRb to the intronic enhancer of *Fra-1*, an event dependent on the conserved MyoD-binding sites located in *Fra-1*.

Recruitment of RNA Polymerase II to *Fra-1* Is Inhibited during Differentiation—We also explored the functional consequences of recruitment of MyoD and pRb to the intronic enhancer of *Fra-1* during differentiation. Various models have been proposed to explain how enhancers operate (56), and in each of them there exists an interaction of the enhancer with the promoter allowing RNA polymerase II (Pol II)² loaded at the enhancer to be transferred to the promoter (43, 56). Accordingly, we predicted that recruitment of MyoD and pRb to *Fra-1* during differentiation might prevent the localization of Pol II to the intronic enhancer of *Fra-1*. C2C12 myoblasts were cultured under conditions to effect a reversible quiescent state or an irreversible differentiated state and then restimulated (Fig. 1). We found that in restimulated quiescent myoblasts (*i.e.* conditions in which *Fra-1* is induced), Pol II was localized to the

intronic enhancer of *Fra-1*, as well as the transcriptional start site (Fig. 7). By contrast, in differentiated myoblasts that were restimulated (*i.e.* conditions where *Fra-1* is not induced), we found that Pol II failed to localize to the intronic enhancer and promoter of *Fra-1* (Fig. 7). As a control, the *MCK* enhancer was analyzed. As anticipated, Pol II failed to localize to the *MCK* enhancer in quiescent and restimulated myoblasts. By contrast, differentiated myoblasts displayed localization of Pol II to the *MCK* enhancer. That localization of Pol II to the *MCK* enhancer was not observed when differentiated myoblasts were restimulated was expected, because this scenario has been shown to result in the suppression of *MCK* mRNA levels (57). These observations suggest that the intronic enhancer of *Fra-1* is rendered inactive with respect to Pol II recruitment upon differentiation of myoblasts.

DISCUSSION

Although it is accepted that MyoD and pRb have essential roles in establishing and perhaps maintaining terminal cell cycle arrest during myogenesis, whether and how they cooperate and the specific events they govern during this process have been unclear. We have provided evidence that MyoD and pRb cooperate to block the induction of cyclin D1, an inhibitor of both MyoD and pRb, during restimulation of differentiated myoblasts. However, the means by which MyoD and pRb

² The abbreviation used is: Pol II, RNA polymerase II.

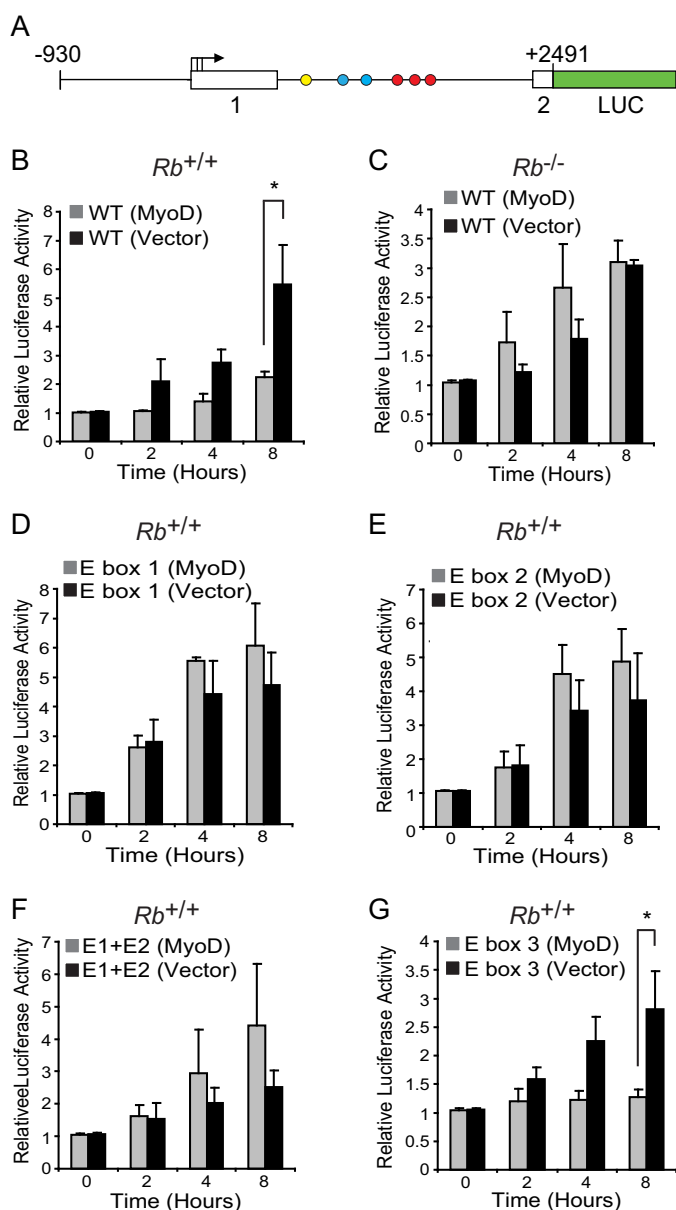


FIGURE 5. Block to *Fra-1* induction by MyoD is mediated through conserved E boxes located in the intronic enhancer of *Fra-1*. *A*, schematic diagram of *Fra-1* promoter reporter with intronic E boxes (blue circles), AP-1 elements (red circles), and a nonconserved E box (yellow circle) indicated. *B* and *C*, block to *Fra-1* promoter induction is pRb-dependent. *Rb*^{+/+} (*B*) and *Rb*^{-/-} (*C*) fibroblasts with a stably integrated wild-type *Fra-1* promoter reporter were infected with MyoD-encoding virus or empty vector and cultured under conditions to induce differentiation. Subsequently, cells were restimulated, and promoter activity was normalized for protein content determined at the times indicated. Two independent sets of promoter reporter lines were made, and each yielded similar results. The results are means \pm S.D. of three independent experiments, each with triplicate determinations. *, *p* = 0.007. *D–G*, block to *Fra-1* promoter induction is dependent on conserved E boxes located in intron 1. The results shown are as in *B*, except E box mutants of the *Fra-1* promoter reporter were analyzed in *Rb*^{+/+} myoblasts. Mutants included were the individually mutated conserved E boxes, E box 1 (*D*), E box 2 (*E*), the double mutant, E box 1 and E box 2 (E1 + E2) (*F*), and a nonconserved E box, E box 3 (*G*). Two independent sets of promoter reporter lines were made, and each yielded similar results. The results are means \pm S.D. of three independent experiments, each with triplicate determinations. *, *p* = 0.032.

impose a block to cyclin D1 induction is indirect. Our findings suggest that together MyoD and pRb render the *Fra-1* gene, an important upstream regulator of cyclin D1 expression, refrac-

tory to induction. These findings establish a previously unappreciated functional link between a lineage-specific transcription factor, a tumor suppressor, and a proto-oncogene. Further, they provide a mechanism by which MyoD can inhibit transcription and suggest that this function of MyoD contributes to an important component of the myogenic differentiation program.

Previous studies established that MyoD is recruited in a temporal fashion to a large cohort of genes during myogenesis, allowing for the orchestration of the differentiation program (18, 22). Several genes affected directly by MyoD are activated; however, a subset of these genes was repressed, and our findings bear on how MyoD might effect transcriptional repression and the biological significance of this phenomenon. The *cis*-regulatory elements of many, but not all genes activated by MyoD, contain two E boxes, and this allows for cooperative binding of MyoD (58). The same is likely occurring at the intronic E boxes of *Fra-1*, because the two conserved E boxes are required for silencing of this gene (Fig. 5). These observations suggest that recruitment of MyoD to intron 1 of *Fra-1* is the initiating event affecting gene expression. At a global level, the effect of MyoD on the *Fra-1* gene occurs early during differentiation; this follows from the observation that *Fra-1* is among the genes most rapidly repressed during myogenic differentiation (19, 20). Thus, the timing of recruitment of MyoD to the *Fra-1* gene appears to effect two outcomes. First, the early localization of MyoD at the *Fra-1* gene brings about the repression of *Fra-1* during the initial phase of the myogenic differentiation program, thereby decreasing the expression of cyclin D1, which in turn allows the appropriate functioning of MyoD. In this regard, it is noteworthy that the kinetics of repression of *cyclin D1* is delayed compared with *Fra-1* (19), consistent with our data suggesting that loss of *Fra-1* expression is causally related to the effect on cyclin D1 expression. Second, recruitment of MyoD effects the stable repression of the *Fra-1* gene, rendering it, and consequently *cyclin D1*, immune to growth factor stimulation. Again, by preventing the induction of cyclin D1, this allows MyoD and other myogenic factors to maintain the mature differentiated characteristics of myotubes, because perturbing the function of myogenic transcription factors is thought to allow dedifferentiation and re-entry into the cell cycle (59).

Although it is generally agreed that pRb is essential for the establishment of an irreversible cell cycle arrest during myogenesis, attempts to determine whether pRb is required for maintenance of terminal cell cycle arrest have led to contradictory results. Specifically, inactivation of a conditional *Rb* allele in terminally differentiated myoblasts followed by serum restimulation failed to induce entry into the S phase (60, 61). Others, using a different strategy, RNAi, to deplete pRb in terminally differentiated myoblasts arrived at the opposite conclusion (62). We believe that our findings bear on this issue. The kinetics of down-regulation of *Fra-1* and *cyclin D1* during myogenesis (19, 20) together with our data suggest that the cooperative actions of MyoD and pRb at *Fra-1* is an early event during the myogenic differentiation program. This suggests that the epigenetic regulation effected by MyoD and pRb at *Fra-1* is stable and participates in the maintenance of the stable repres-

pRb and MyoD Function during Terminal Cell Cycle Arrest

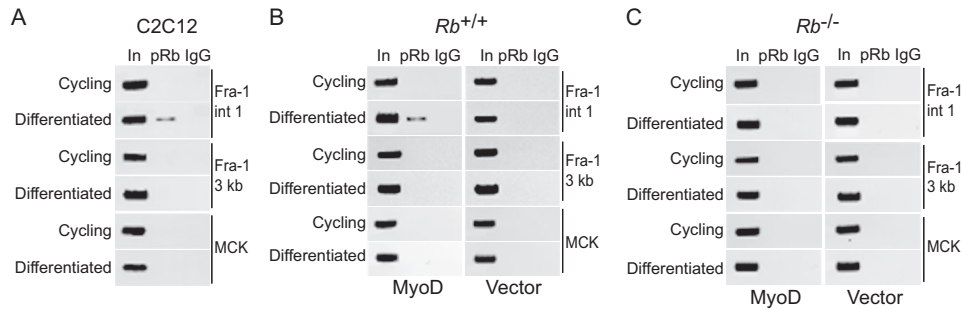


FIGURE 6. pRb recruitment to the intronic enhancer of *Fra-1* is MyoD-dependent. *A*, pRb is recruited to intron 1 of *Fra-1* during differentiation. ChIP analysis of cycling and differentiated C2C12 cells was performed with antibody to pRb or IgG as control. Sequences spanning the E boxes in intron 1 of *Fra-1* (Fra-1 int 1), ~3 kb downstream of the *Fra-1* intronic E boxes (Fra-1 3 kb), and the *MCK* enhancer (*MCK*) were analyzed by PCR. One-tenth of the lysates used for ChIP were also subjected to PCR (input, *In*). The results are representative of at least three independent experiments. *B*, pRb recruitment to intron 1 of *Fra-1* is MyoD-dependent. The results shown are as in *A*, except *Rb*-positive fibroblasts were infected with MyoD-encoding virus (left panel) or empty vector (right panel). The results are representative of at least three independent experiments. *C*, as in *B*, except *Rb*-deficient fibroblasts were employed. The results are representative of at least three independent experiments.

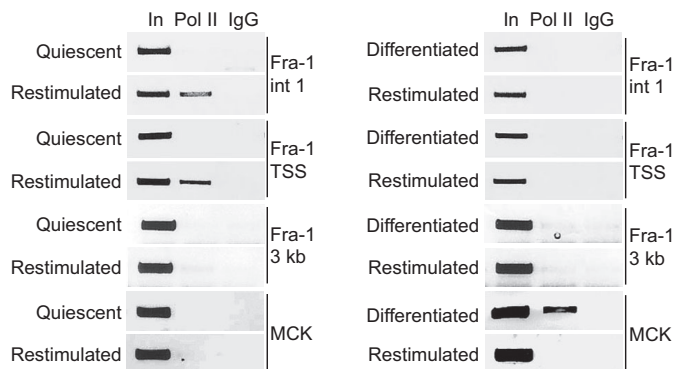


FIGURE 7. Block to RNA polymerase II recruitment to *Fra-1* during differentiation. C2C12 cells were rendered quiescent (left panel) by culturing in 0.1% FBS for 3 days or differentiated (right panel) by culturing in 2% horse serum for 6 days. Subsequently, the cells were restimulated for 8 h in the presence of 20% FBS. ChIP analysis was performed with antibody to Pol II or IgG as control. Sequences spanning the E boxes in intron 1 of *Fra-1* (Fra-1 int1), the transcriptional start site of *Fra-1* (Fra-1 TSS), ~3 kb downstream of the *Fra-1* intronic E boxes (Fra-1 3 kb), and the *MCK* enhancer (*MCK*) were analyzed by PCR. One-tenth of the lysates used for ChIP were also subjected to PCR (input, *In*). The results are representative of at least two independent experiments.

sion of *Fra-1* and, in turn, *cyclin D1*, although it cannot be ruled out that other events independent of MyoD and pRb are involved (see below). Consistent with these possibilities, cyclin D1 is not induced upon restimulation of differentiated myoblasts in which *Rb* has been inactivated (61).

The ability of MyoD to induce the cdk inhibitor p21 is often regarded as the mechanism underlying irreversible cell cycle arrest of differentiated myoblasts (27, 63, 64). However, p21 is induced by MyoD in *Rb*-deficient myoblasts but fails to bring about terminal cell cycle arrest in this setting (8), and ectopic expression of p21 in *Rb*^{-/-} myoblasts fails to inhibit DNA synthesis (8). Further, p21, like p16, would be predicted to effect a late G₁ arrest during restimulation of differentiated myoblasts. However, previous studies have suggested that restimulated differentiated myoblasts are blocked in mid G₁ (46), and our findings indicate that an important component of the immediate early response is inhibited, suggesting the block is in early G₁. Based on these observations, we suggest a role for p21 induction in effecting the accumulation of unphosphorylated active pRb, thereby facilitating its ability to participate in the block to *Fra-1* expression.

How MyoD is recruited to the intronic E boxes of *Fra-1* as a function of differentiation is not known, but this also remains to be determined for muscle-specific genes containing two E boxes. The localization of MyoD to intron 1 of *Fra-1* allows for the recruitment of pRb (Fig. 6). The interaction between MyoD and pRb has been reported (25) and reproduced by others (65), but nonetheless remains controversial (29, 65). This has largely been due to the inability to place the interaction between pRb and MyoD into a physiological setting of one or more of the events that occur during myogenesis. For example, although pRb is clearly required for the ability of MyoD to induce *MCK* expression, the contribution of pRb in this setting is indirect (6, 15), consistent with our finding that pRb is not recruited to this gene (Fig. 6). Thus, there does not appear to be a motivation or need to invoke an interaction between pRb and MyoD in effecting myogenic differentiation independent of their role in terminal cell cycle arrest. Our findings suggest that the interaction between pRb and MyoD does occur, but in a gene-specific manner to effect terminal cell cycle arrest. How this promoter/enhancer selectively occurs remains to be determined. An attractive hypothesis might be that MyoD-pRb complexes are stabilized at intron 1 of *Fra-1* by a factor already present at the *Fra-1* gene, similar to what has been shown for the homeodomain protein Pbx (44). Related to this possibility, it is noteworthy that the intronic enhancer in *Fra-1* is embedded within a CpG island, suggesting a possible role for the context of the sequence in which the MyoD-binding sites in *Fra-1* are located. Regardless of the details, our findings suggest a physiological context in which the cooperative actions of MyoD and pRb during myogenesis congregate at intron 1 of *Fra-1*.

Previous studies, albeit conflicting, have suggested that histone modifications at the *cyclin D1* promoter render it refractory to restimulation. In one study, it was suggested that methylation of lysine 9 in histone H3 (H3K9me3) was responsible for the repression of *cyclin D1* (66). By contrast, another group failed to detect H3K9me3 at the *cyclin D1* promoter in differentiated myoblasts and suggested that the disparate results may be due to the use of improved reagents (62). Rather, they found that *cyclin D1* was marked by H3K27me3 in differentiated myoblasts and that this occurred even in the absence of pRb (62), suggesting an event independent of the cooperative actions of

MyoD and pRb described here. It is noteworthy, however, that a promoter marked by H3K27me3 is not necessarily inactive if it is bivalent, *i.e.* marked by both H3K4me3 and H3K27me3 (67, 68). Along these lines, in embryonic fibroblasts, similar to one of the experimental systems employed here, *cyclin D1* displays bivalent histone marks (68). This suggests that although *cyclin D1* harbors the repressive H3K27me3 mark, it may not be sufficient to silence *cyclin D1*. Further, our finding that ectopic expression of Fra-1 in differentiated myoblasts is capable of inducing cyclin D1 to a level seen in cycling myoblasts (Fig. 3), suggests that *cyclin D1* is not rendered refractory to induction during the state of differentiation. Here, it is noteworthy that C2C12 cell ChIP-seq data at the ENCODE Project at UCSC reveals localization of Fra-1 to the *cyclin D1* promoter, suggesting a direct effect of Fra-1. Further studies will be required to determine how MyoD and pRb affect the activity of the intronic enhancer of *Fra-1* to, for example, influence the recruitment of RNA polymerase II and whether and how the bivalent state of *cyclin D1* is resolved during differentiation. Notwithstanding, we cannot rule out the possibility that methylation events at *cyclin D1* might facilitate its silencing during the maintenance of a terminally differentiated state in an pRb-independent manner.

Our findings suggest that pRb and MyoD, by imposing a block in early G₁, prevent S phase re-entry of differentiated myoblasts. This is distinct from fibroblasts exiting quiescence where pRb acts in late G₁ at a time that temporally and functionally coincides with the restriction point (49). However, in both settings, cyclin D appears to be the target: in fibroblasts, p16, a cyclin D-cdk4/6 inhibitor, effects an pRb-dependent late G₁ block, and restimulation of differentiated myoblasts does not progress past early G₁ because cyclin D1 is not synthesized. Why restimulated differentiated myoblasts choose to effect their arrest in G₁ prior to the synthesis of cyclin D1 is not clear but can perhaps be rationalized. Cyclin D1 can inhibit several myogenic transcription factors including MyoD (see Introduction); thus, blocking the synthesis of cyclin D1 is necessary for the establishment and maintenance of the differentiated phenotype. Hence, it appears that the timely coordination of terminal cell cycle arrest with differentiation is not just coincidence but rather a necessary component of the myogenic differentiation program. Further, *Fra-1* is a proto-oncogene (52), and it is likely that cyclin D1 does not represent its sole effector of transformation and that other targets of Fra-1 are deleterious to myogenesis. In this context, it is noteworthy that ectopic expression c-Jun, the product of another immediate early gene and heterodimeric partner of Fra-1, inhibits myogenesis (69). Regardless, the choice to target *Fra-1*, as opposed to *cyclin D1*, may reflect *Fra-1* as being a nodal point whose expression must be blocked for the appropriate execution of differentiation.

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