Activation of Cdc6 by MyoD is associated with the expansion of quiescent myogenic satellite cells

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MyoD is a transcriptional factor that is required for the differentiation of muscle stem cells (satellite cells). In this study, we describe a previously unknown function for MyoD in regulating a gene (*Cdc6*) that is vital to endowing chromatin with the capability of replicating DNA. In C2C12 and primary mouse myoblasts, we show that MyoD can occupy an E-box within the promoter of *Cdc6* and that this association, along with E2F3a, is required for its activity. MyoD and Cdc6 are both expressed after quiescent C2C12 myoblasts or satellite cells in association with myofibers are stimulated for growth, but MyoD appears at least 2–3 h earlier than Cdc6. Finally, knockdown of MyoD impairs the ability of C2C12 cells to express Cdc6 after leaving quiescence, and as a result, they cannot fully progress into S phase. Our results define a mechanism by which MyoD helps myogenic satellite cells to enter into the first round of DNA replication after transitioning out of quiescence.

Introduction

Satellite cells are self-renewing muscle stem cells, which mediate the growth and repair of skeletal muscle (Zammit et al., 2006). When the muscle is damaged, these cells leave their state of quiescence, proliferate at length to generate colonies of myoblasts, and then differentiate to form multinucleated myotubes (Zammit et al., 2006). The myogenic factor that is mainly involved in controlling the progression of these myoblasts into the differentiation state is MyoD (Le Grand and Rudnicki, 2007). This protein, which preferentially binds to a DNA consensus site (CANNTG) called an E-box, has been shown in both myogenic (C2C12) and nonmyogenic cell lines to be highly important in controlling the transcription of differentiation-specific genes (Berkes and Tapscott, 2005). This function correlates in part to the ability of MyoD to recruit histone acetyltransferases (e.g., PCAF and p300) and SWI/SNF chromatin-remodeling complexes to the promoters of these genes to affect specific changes in chromatin structure (Mal and Harter, 2003; Berkes et al., 2004). Another myogenic factor that is highly related to MyoD in both amino acid sequence and affinity to E-box sites is Myf5 (Tapscott, 2005). Mice that are null for either *MyoD* or *Myf5* are viable, and therefore, it is widely believed that these two proteins can

functionally substitute for one another during embryonic development (Le Grand and Rudnicki, 2007). However, this compensation may not be as efficient in the regeneration of muscle because cultures of MyoD$^{-/-}$ myoblasts, which apparently have 5–10 times more Myf5 protein, are dramatically delayed in their transition to a differentiated state (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999). Moreover, these myoblasts also appear to be limited in their ability to expand in population when compared with wild type (Montarras et al., 2000), and this may reflect the fact that Myf5 is less effective than MyoD in regulating the same set of growth-phase genes (Montarras et al., 2000; Ishibashi et al., 2005).

There is evidence to suggest that MyoD may also be playing a role in helping satellite cells to expand in population upon leaving quiescence. For example, MyoD has been shown to be expressed within 3 h after the satellite cells have been activated (Jones et al., 2005). In addition, ChIP (chromatin immunoprecipitation)-on-chip analysis has shown that MyoD can target genes other than those involved in the myogenic program, and in this group were *Cdc6* and *MCM2*. These two genes are primarily involved in making chromatin operational for DNA replication, a function which is not only essential for the proliferation of cells but also

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; DM, differentiating medium; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GM, growth medium; shRNA, short hairpin–mediated RNAi.

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Figure 1. **MyoD occupies the *Cdc6* promoter in myoblasts.** (A) Schematic of the *Cdc6* promoter. Closed ovals (E1 and E2) and squares identify the putative MyoD (E-boxes) and E2F sites within the promoter, respectively. The inverted arrows represent the primers used in the PCR amplification reactions. The transcription start site is depicted with an arrow. (B) Cross-linked chromatin from C2C12 myoblasts or primary mouse myoblasts cultured in GM was immunoprecipitated in parallel with anti-MyoD antibody (Ab) and then analyzed by semiquantitative PCR using primers surrounding the E1 and E2 sites in the *Cdc6* promoter. A control reaction with the use of normal rabbit IgG (NR IgG) is shown along with input DNA (0.05%), which was amplified by the same set of primers. Black lines indicate that intervening lanes have been spliced out. (C) ChIP experiments were performed in parallel on chromatin from C2C12 myoblasts before and after differentiation using normal rabbit IgG or an antibody specific for MyoD. Precipitated DNA was then analyzed by PCR using the E1 primers, and afterward, the bands were quantified by ImageJ (version 1.36b; National Institutes of Health). Controls for PCR included the use of normal rabbit IgG and the titration of input DNA to ensure all amplifications were within the linear range (Fig. S1 D). (D) Values in the histogram represent the ratio of chromatin-bound MyoD to input genomic DNA and are the mean of three independent experiments with standard deviation.
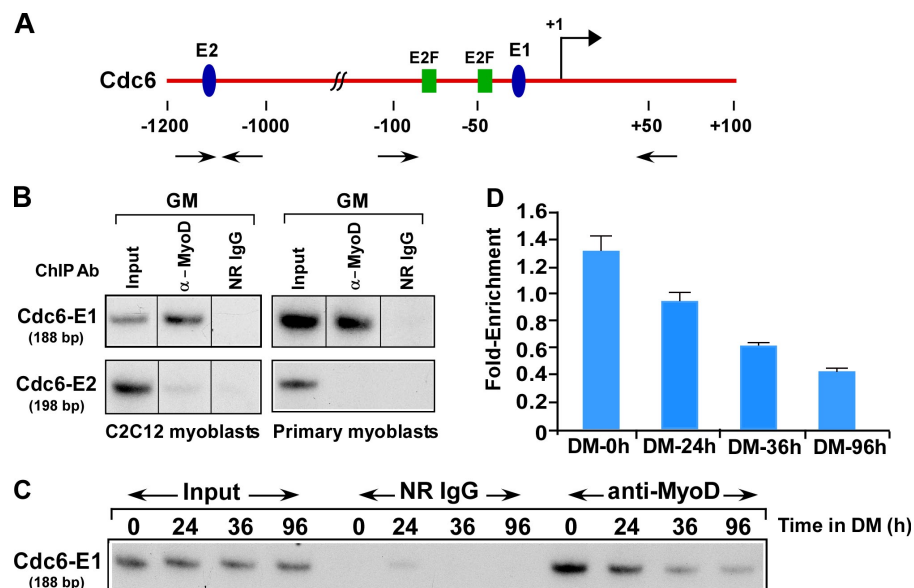
for cells emerging from quiescence (Blais et al., 2005; for review see Machida et al., 2005). MCM2 is a member of the MCM family of proteins (MCM2–7), and as a complex, these proteins along with Cdc6 and at least seven other proteins assemble at origins of DNA replication to form what is called a prereplicative complex. Studies have shown that Cdc6 is a key participant in this process because it essentially loads the MCM proteins onto the origins (for review see Machida et al., 2005). Because prereplicative complexes are largely involved in unwinding the DNA and initiating the process of replication, it is perhaps not surprising to find that the genes of many of its members, including *Cdc6* and *MCM2*, have become temporarily silent in quiescent cells (Yan et al., 1998; Ren et al., 2002; Ghosh and Harter, 2003). Although this scenario has yet to be demonstrated in satellite cells, it is most likely that analogous mechanisms would occur, and if so, it is possible that MyoD could be involved in inducing transcription from either *Cdc6* or *MCM2* in these cells after leaving quiescence.

In this study, we have used mouse primary myoblasts (wild type or MyoD$^{-/-}$), isolated myofibers with associated satellite cells, and the satellite-derived C2C12 cell line to investigate whether MyoD can possibly regulate genes that are an integral part of the replication system. Through biochemistry and RNAi experiments, we have uncovered a mechanism that involves MyoD in initiating the expression of *Cdc6* in growth-stimulated quiescent myoblasts and shown that this induction is essential for effectively moving these cells into S phase. Furthermore, the importance of MyoD's activity in this context is underscored by Myf5's ability to perform this function in the absence of MyoD, although with less efficiency. Thus, our results have established a new and unexpected role for MyoD, and by all accounts, one that may be important to satellite cells as they transition out of quiescence.

Results

Analysis of promoters for potential E-box sites

To identify potential MyoD-binding sites in the regulatory regions of genes that have been identified in inducing DNA replication, we used TRANSFAC (version 7.0, cut off 85), a database which models factor-site interactions on the sequences of DNA (Matys et al., 2006). With this computer-assisted analysis, we were able to identify within the promoters of *Cdc6* and *MCM2* and *-7* a consensus DNA site known as an E-box for the binding of MyoD (Fig. S1 A and not depicted). The *Cdc6* promoter, the one we chose to focus on, was found to contain two of these sites, with one (E1) being in close proximity to the transcriptional start site (+1) and the other (E2) much further upstream (Fig. 1 A). The position of the start site within this promoter corresponds to the 5' terminus of an mRNA (Materials and methods) that is predicted to encode a protein of ~62 kD, and in fact, a protein matching this size can be observed in extracts of proliferating C2C12 myoblasts, as judged by Western blot analysis (Fig. S1 B). The regulatory region of *Cdc6* also contains two potential E2F-binding sites close to the transcription start site, and interestingly, one of them is located 10 bp upstream from the E1 E-box (Fig. 1 A). The arrangement of these two E2F elements is quite similar to that found in the human *Cdc6* promoter (Williams et al., 1997), and it has been reported that the one nearest to the start site is mainly involved in stimulating its activity in nonmuscle cells (Schlisio et al., 2002).

Both MyoD and E2F can occupy the promoter of *Cdc6* in myoblasts

We used the myogenic cell line C2C12 and mouse primary myoblasts to determine by ChIP whether any of the E-boxes within the endogenous *Cdc6* promoter were capable of associating

A

B

C

D
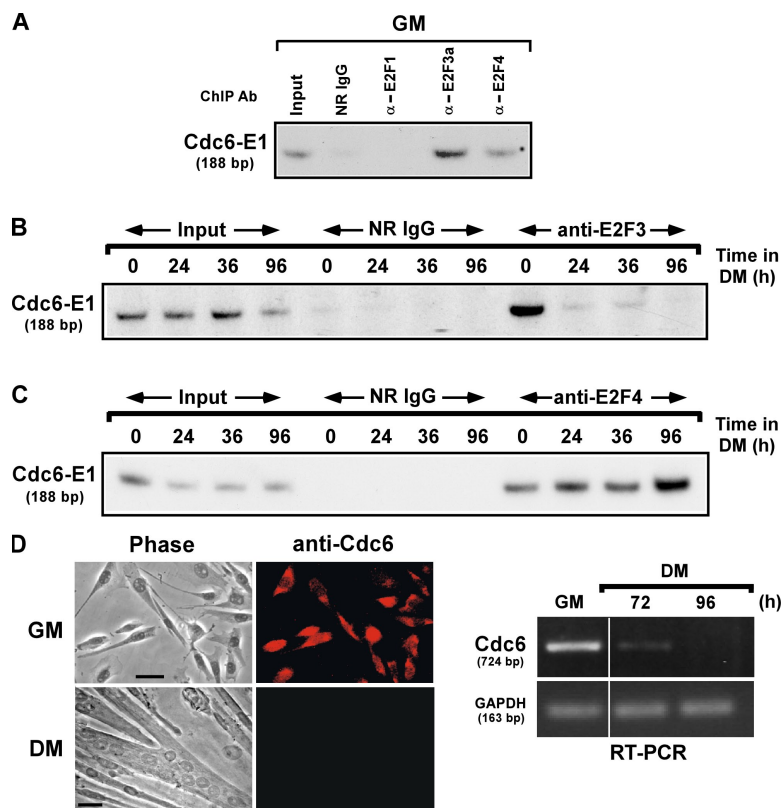
Figure 2.   **E2F3a occupies the *Cdc6* promoter in myoblasts, but it is replaced by E2F4 after differentiation.** (A) ChIP of C2C12 myoblasts cultured in GM with antibodies (Ab) detecting E2F1, E2F3a, and E2F4. As a control, ChIP was also performed in parallel with normal rabbit IgG (NR IgG). Purified DNA from enriched chromatin fragments was amplified by PCR with the E1 primers. (B and C) ChIP of C2C12 myoblasts cultured in DM at the indicated times using normal rabbit IgG and either anti-E2F3a or anti-E2F4, respectively. Precipitated DNA was analyzed for the presence of the *Cdc6* promoter by PCR. (D) The occupation of the *Cdc6* promoter by E2F4 correlates with its silencing in differentiated myoblasts. Immunofluorescent staining of C2C12 myoblasts cultured in GM or DM for 96 h was performed by using a Cdc6-specific antibody. Antibody staining is visualized as red. RT-PCR detection of Cdc6 transcripts in C2C12 myoblasts cultured in GM or in DM for 72 or 96 h. GAPDH expression is used as a loading control. White lines indicate that intervening lanes have been spliced out. Bars, 20 µm.

with MyoD. After immunoprecipitating cross-linked DNA fragments of ~100–400 bp with antibodies specific for MyoD, we found that MyoD could associate with this promoter in both of these cellular environments but only near the E1 site (Fig. 1 B). The interaction of MyoD with this promoter was not the result of a promiscuous interaction because it was also able to occupy the downstream and upstream regulatory regions of *MCM2* and -7, respectively (Fig. S1 C and not depicted; Juven-Gershon et al., 2008). ChIP assays in combination with kinetic experiments were then used to determine whether MyoD remained bound to the *Cdc6* promoter after the myoblasts were induced to differentiate. As shown in Fig. 1 (C and D), the interaction of MyoD with this promoter diminished over time as myoblasts underwent the process of differentiation. This result is significant because it indicates a requirement for MyoD in regulating the expression of *Cdc6* in satellite cells, but only while the cells are in a state of proliferation.

ChIP experiments were also performed to determine whether any member of the E2F family of transcription factors (E2F1–7) might be interacting with the *Cdc6* promoter in proliferating myoblasts. As shown in Fig. 2 A, E2F3a, an isoform mainly expressed in growing cells (Leone et al., 2000), was found to be the most predominant factor in association with this promoter, although a weak binding by E2F4 could also be observed. Note that the near absence of E2F4 from the *Cdc6* promoter was not the result of an antibody that had lost its specificity when used in a ChIP assay (Fig. 2 C). Although the site on the promoter to which E2F3a is binding has yet to be identified, it is possible that it may be the one closest to the transcriptional start site (Fig. 1 A), as has been previously

shown in the human *Cdc6* promoter (Schlisio et al., 2002). We also observed in this analysis that E2F1 was noticeably absent from the *Cdc6* promoter (Fig. 2 A), and again, this result is consistent with what has been previous reported for the human *Cdc6* promoter (Takahashi et al., 2000; Ren et al., 2002; Schlisio et al., 2002). E2F3a has been reported to play an important role in controlling genes that are critical to the replication of DNA. In fact, the ability of cells to enter S phase after being released from a G1/S block becomes markedly diminished if E2F3a is purposely made inactive (Leone et al., 1998). Because of this and the fact that the cessation of DNA synthesis is concurrent with the differentiation of muscle cells, we examined by ChIP assays the relationship between E2F3a and the *Cdc6* promoter after myoblasts were induced to differentiate. We found by temporal analysis that E2F3a could no longer be detected on the *Cdc6* promoter after 24 h of differentiation (Fig. 2 B). Perhaps equally important is that this loss also corresponded to a linear increase in the association of E2F4 with this promoter (Fig. 2 C). This observation and the fact that E2F4 functions primarily as a repressor of transcription (Blais et al., 2005) may be one reason why the expression of *Cdc6* is no longer seen in differentiated muscle cells, either by immunofluorescence or by RT-PCR analysis (Fig. 2 D). We note that Cdc6 is primarily located in the nucleus of proliferating myoblasts, with some evidence of it in the cytoplasm (Fig. 2 D). This phenomenon has also been observed by others (Petersen et al., 1999; Delmolino et al., 2001) presumably because Cdc6 remains chromatin bound after S phase is initiated so that it can then participate in other cellular functions such as mitotic entry (Clay-Farrace et al., 2003).

Figure 3. The E1 and E2F sites are functionally important to the activity of the *Cdc6* promoter. (A) A schematic representation of three luciferase (Luc) reporter constructs, empty vector (pGL3-basic), and a construct driven by a wild-type (wt) *Cdc6* promoter (Cdc6-wt) or a *Cdc6* promoter harboring mutations within the E1 site (Cdc6-mut1). (B) C3H10T1/2 cells were separately transfected with empty vector and with Cdc6-wt or Cdc6-mut1, either alone or with increasing amounts of a plasmid encoding MyoD (pCMV-MyoD). Activity is expressed as fold increase in comparison with activity observed with empty vector after correcting for transfection efficiency (see Materials and methods). (C) Transcriptional-activity of the empty vector or the Cdc6-luciferase constructs in C2C12 myoblasts with or without mutations within E1, E2F, or both sites. (B and C) Error bars represent SEM.
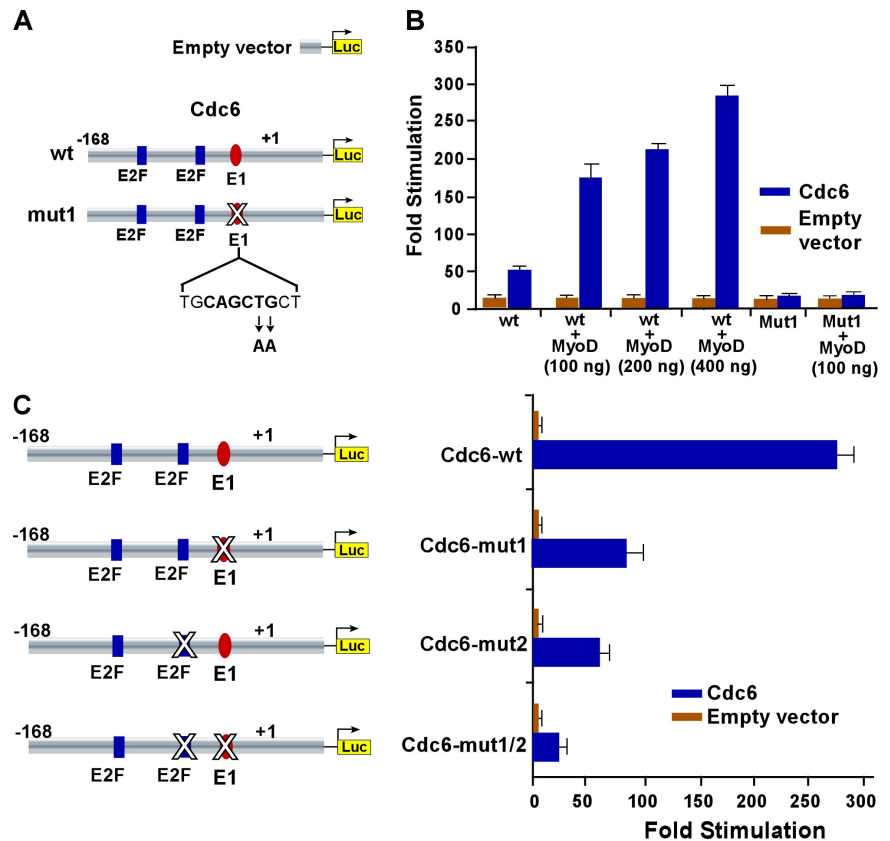
The proximal promoter of *Cdc6* is responsive to MyoD, Myf5, and E2F

To determine whether the E1 site was functionally important to the activity of the *Cdc6* promoter, we first assessed the response of a reporter construct (Cdc6-wt) carrying the regulatory elements of *Cdc6* in a cell line (C3H10T1/2) that does not express endogenous MyoD protein (Fig. 3 A). After this vector and a vector devoid of the *Cdc6* elements were separately transfected into these cells, we found that the promoter activity of Cdc6-wt was enhanced at least sixfold when compared with the empty vector (Fig. 3 B). More importantly, though, this level of activity, which may be caused by an E2F (Fig. 2 A and Fig. S1 E), increased significantly in a dose-dependent manner after the Cdc6-wt vector was cotransfected with a vector expressing increasing amounts of MyoD (pCMV-MyoD; Fig. 3 B). To verify that this effect was truly a function of MyoD, we introduced into the E1 site of the Cdc6-wt vector a two-base mutation that has been previously shown to eliminate the binding of MyoD (Fig. 3 A; Lee et al., 2001). After transfecting this mutant vector (Cdc6-E1mut) either alone or together with pCMV-MyoD into C3H10T1/2 cells, we found that the promoter activity of Cdc6-E1mut was now severely compromised when compared with that of the Cdc6-wt vector (Fig. 3 B).

Microarray analysis has established that Myf5 can regulate many of the same genes as MyoD in proliferating cells (Ishibashi et al., 2005). Therefore, we examined the potential responsiveness of the Cdc6-wt reporter to Myf5 in C3H10T1/2 cells. After Cdc6-wt was introduced into these cells along with a plasmid encoding Myf5 (pCMV-Myf5), we found that the

activity of its promoter had increased substantially when compared with its activity without Myf5 (Fig. S1 F). As expected, the activity of the mutant *Cdc6* promoter (Cdc6-E1mut) was dramatically diminished after it was cotransfected into these cells with pCMV-Myf5 (Fig. S1 F). It would appear then that the proximal promoter region of *Cdc6* is responsive to Myf5 as well, and this may be significant in light of the observations described below (see Fig. 6).

To further investigate the relationship between MyoD and the *Cdc6* promoter and, more importantly, to ensure that the aforementioned results were not associated with an over-expressed MyoD, we introduced each of the reporter constructs into C2C12 myoblasts. As shown in Fig. 3 C, the transfected Cdc6-wt reporter responded to endogenous MyoD by showing a dramatic increase in its promoter activity (an ∼50-fold increase) relative to empty vector. However, the Cdc6-E1mut construct responded by showing at least a threefold reduction in its promoter activity when compared with that of the wild-type vector (Fig. 3 C), thereby lending further support to a role for the E1 site in controlling the *Cdc6* promoter. To determine whether both the E2F and E1 sites were required for precise functioning of the *Cdc6* promoter, we constructed two additional vectors in which the E2F-binding site (Cdc6-mut2) or both the E2F- and E1-binding sites (Cdc6-mut1/2) were altered by mutations (Fig. 3 C). Myoblasts were then separately transfected with each of these vectors. As shown in Fig. 3 C, the promoter activity of the Cdc6 reporter holding a mutation only in the E2F-binding site was significantly reduced when compared with that of wild type. What is more important, though, is that mutations in both

the E2F and E1 sites nearly abolished the activity of the *Cdc6* promoter in these cells (Fig. 3 C). Overall, these experiments suggest that the proximal E1 site within the *Cdc6* promoter is responsive to MyoD, and when considering the results presented in Fig. 2 A, it appears that both MyoD and E2F3a may be required to affect full transcriptional activity of the *Cdc6* gene in muscle cells.

Expression of MyoD and Cdc6 after C2C12 myoblasts or primary satellite cells leave quiescence

Recent studies have identified several genes that are up-regulated as cells move out of quiescence, and of those are a class of which encodes proteins required for giving chromatin a "license" to synthesize DNA (for review see Machida et al., 2005). *Cdc6* is one of those genes (Ren et al., 2002; Ghosh and Harter, 2003), and because our data indicate that MyoD can apparently affect its promoter (Figs. 1–3), we hypothesized that it could be involved in transcriptionally activating *Cdc6* after satellite cells leave quiescence. If this were the case, by all accounts, MyoD ought to be expressed before Cdc6 in these cells. As an initial strategy for investigating this possibility, we induced C2C12 myoblasts into a state that closely mimicked the properties of a quiescent satellite cell. This conversion was accomplished by using a well-established procedure (Kitzmann et al., 1998; Spiller et al., 2002), which includes the use of a methionine-depleted DME supplemented with 1% serum (see Materials and methods). When low-density C2C12 myoblasts were cultured under these conditions for 36 h, we found that at least 95% of them were no longer synthesizing their DNA, as judged by the incorporation of BrdU (Fig. S2 A). What is more important, though, is that these cells were barely expressing MyoD (Fig. S2 B), as indicated by immunofluorescence or RT-PCR analysis. This result strongly indicated that the majority of the myoblasts were now in a non-differentiated and quiescent stem cell state. We also examined the expression of *Cdc6* in the quiescent myoblasts, and as shown in Fig. S2 B, its level of transcription was dramatically reduced as well. These data were not entirely surprising because *Cdc6* was no longer required to prepare chromatin for DNA replication in these cells nor participate in checkpoint mechanisms.

Having the C2C12 myoblasts in a quiescent state enabled us to determine whether there were any kinetic differences in expression between *MyoD* and *Cdc6* once these cells moved into G1. After the quiescent myoblasts were restimulated for growth, we found that *MyoD* was indeed expressed at least 2–3 h earlier than *Cdc6*, as judged by both Western blot and RT-PCR analysis (Fig. 4 A). In this study, the kinetics of E2F3a expression were also monitored, and the profile was found to be virtually indistinguishable from that of *MyoD* (Fig. S2 C). Given this result, it was next important to determine whether the temporal expression observed between MyoD and Cdc6 was also essential to growth-stimulated primary satellite cells. Therefore, quiescent satellite cells in association with myofibers isolated from the muscle of an adult mouse were stimulated with serum and then processed for immunofluorescence using antibodies against Pax7, MyoD, or Cdc6. The staining for Pax7 was performed in this case to mark both the quiescent and activated
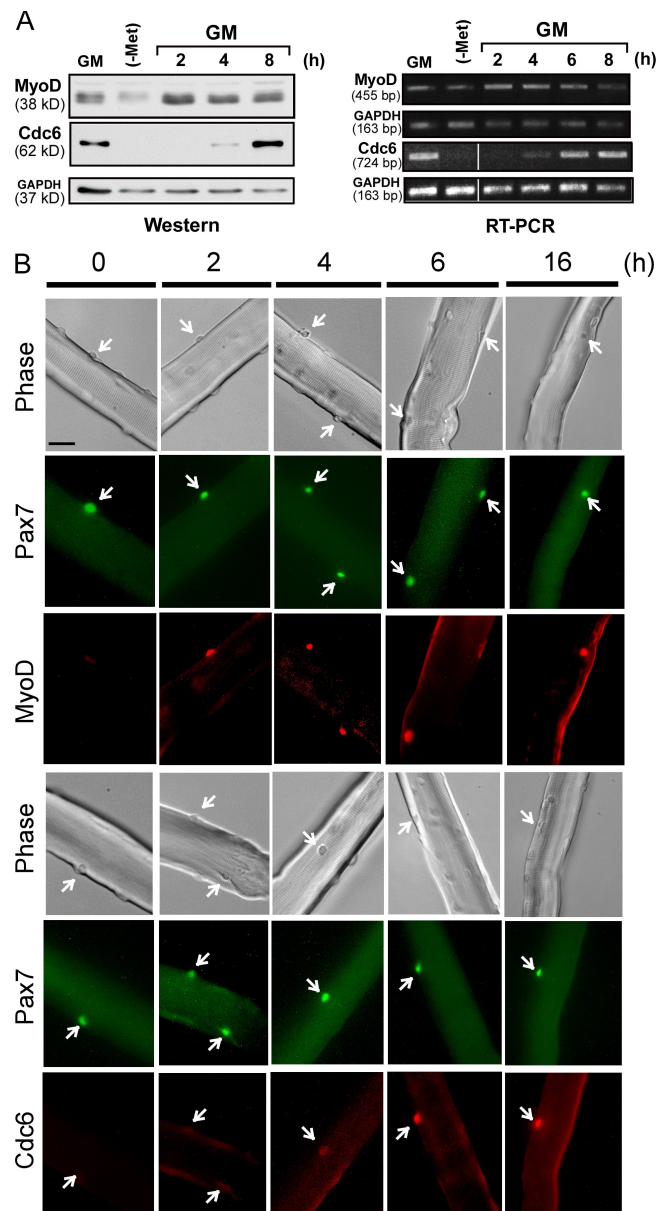

Figure 4. **MyoD is expressed before Cdc6 after C2C12 myoblasts or primary satellite cells transition from quiescence.** (A) C2C12 myoblasts were cultured either in GM or in methionine-free medium (−Met) with 1% serum for 36 h and then restimulated with GM for the indicated times. For Western blot analysis, whole cell extracts were prepared, resolved on SDS-PAGE, and then probed with antibodies specific to MyoD, Cdc6, and GAPDH. MyoD and Cdc6 mRNAs were measured by RT-PCR. GAPDH was used as the internal control. White lines indicate that intervening lanes have been spliced out. (B) Isolated extensor digitorum longus myofibers were cultured in basal medium (0 h) before switching to mitogen-rich medium for the activation of satellite cells. The myofibers were fixed at the indicated times and then coimmunostained for Pax7 (green) and MyoD (red) or Cdc6 (red). Immunostaining for Pax7 identifies the satellite cells (Buckingham, 2007). Arrows point to the individual satellite cells. Approximately 200 myofibers were analyzed in this experiment. Bar, 20 µm.

satellite cells (Buckingham, 2007). As shown in Fig. 4 B, after the satellite cells were serum stimulated and then costained for Pax7 and MyoD or Pax7 and Cdc6, we found that the expression of *MyoD* was occurring at least 2–3 h before the expression of *Cdc6* (Fig. 4 B). We also observed in this experiment that the

Figure 5. **Knockdown of MyoD dramatically reduces the expression of Cdc6 in growth-stimulated quiescent myoblasts.** (A) C2C12 myoblasts were separately transduced with lentiviruses expressing two distinct shRNAs specific for MyoD (shMyoD-64 and -66) and a scramble shRNA (shCntrl). After 42 h, the myoblasts were brought to quiescence and then stimulated to grow for the indicated times in GM. (B and C) After stimulation, the myoblasts were harvested, and the expression of MyoD or Cdc6 was then monitored either by RT-PCR (B) or by Western blotting (C). NS denotes a nonspecific band. EV, empty vector. (D and E) Cross-linked chromatin was isolated from growth-stimulated quiescent myoblasts expressing a scramble shRNA (shCntrl) or an shRNA specific for MyoD (shMyoD-66). Afterward, the protein–DNA fragments were immunoprecipitated in parallel with anti-E2F3a or anti-MyoD antibody (Ab) and then analyzed by semiquantitative PCR using primers surrounding the E1 site within the *Cdc6* promoter. A control reaction with the use of normal rabbit IgG (NR IgG) is shown along with input DNA, which was amplified by the same set of primers. Black lines indicate that intervening lanes have been spliced out.
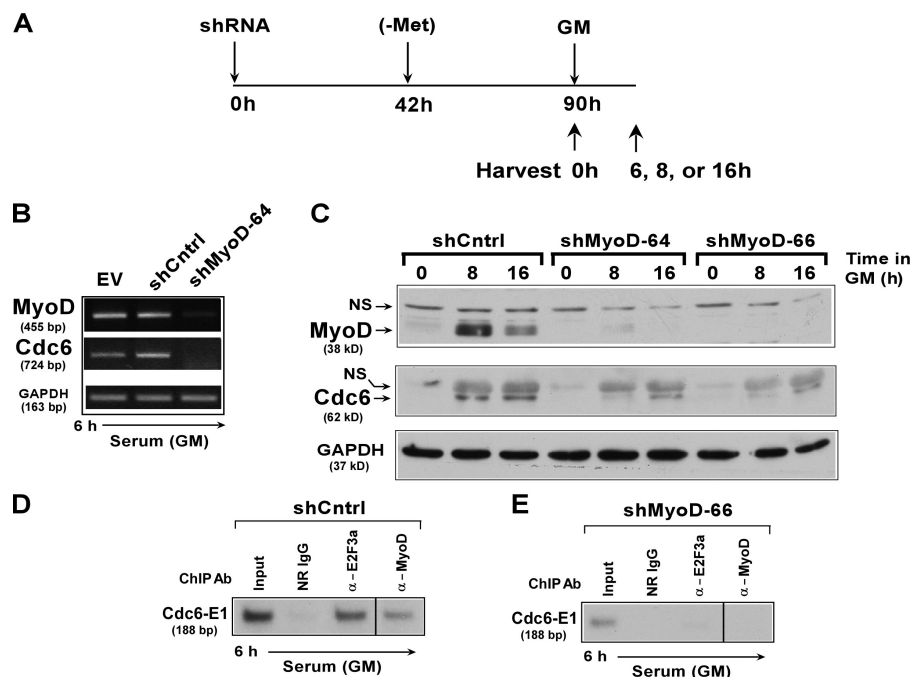
activated satellite cells were coexpressing MyoD and Cdc6 by at least 8 h (Fig. S2 D). Notably, these results are indistinguishable from those observed in the growth-stimulated quiescent C2C12 myoblasts (Fig. 4, A and B), and together with the data shown in Fig. 3, they strongly suggest that the expression of *Cdc6* in newly activated satellite cells may be regulated by *MyoD*.
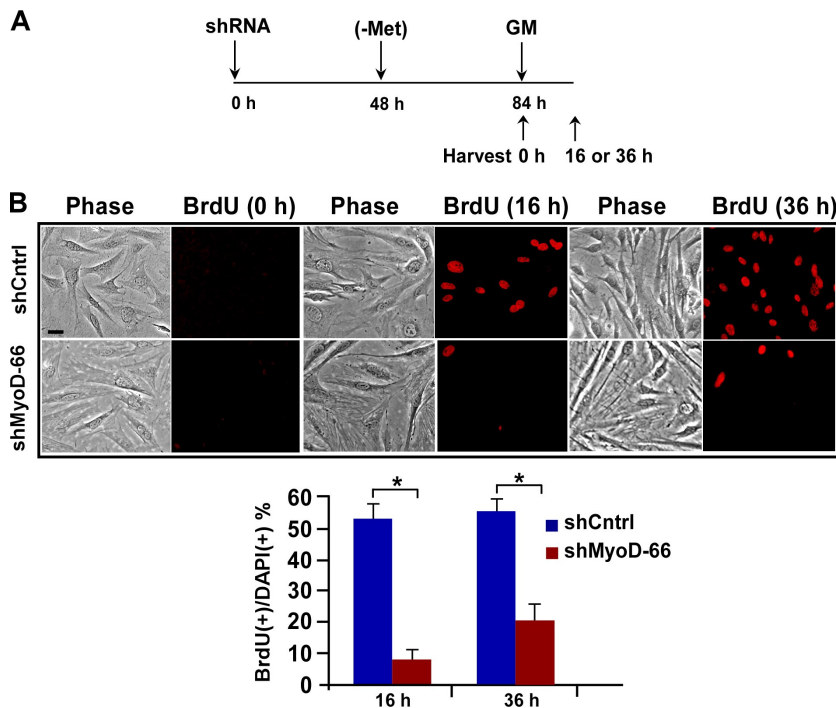
We also examined the status of Myf5 expression in both quiescent and serum-stimulated C2C12 myoblasts. In contrast to MyoD, we found Myf5 to be highly expressed in the quiescent myoblasts, as determined by Western blot analysis (Fig. S2 C). However, after these cells reentered the cell cycle, its expression sharply declined, but only to resume at ∼8 h into the cell cycle (Fig. S2, C and E; and not depicted). This pattern of Myf5 expression as myoblasts leave quiescence has been previously reported by others (Kitzmann et al., 1998) and models, to some extent, the activity of the *Myf5* locus in both quiescent and newly activated satellite cells (Le Grand and Rudnicki, 2007).

MyoD is required for the induction of Cdc6 after myoblasts transition out of quiescence

As shown in Figs. 3 and 4, our results suggested that *MyoD* participates in the activation of *Cdc6* after myogenic progenitors are stimulated to leave quiescence. To further investigate this possibility, we used short hairpin–mediated RNAi (shRNA) to knock down the expression of MyoD in the hope of determining what effect, if any, this would have on the induction of Cdc6 after quiescent myoblasts were returned to the cell cycle. Three shRNAs (shRNA-63, -64, and -66) were designed to specifically target the coding sequences of MyoD, and in the end, shRNA-64 and -66 were found to be the most effective in knocking down the expression of MyoD (∼85–90%) when compared with a scrambled shRNA

(unpublished data). Next, lentiviral vectors expressing shRNA-64 (shMyoD-64), shRNA-66 (shMyoD-66), or the scrambled shRNA (shCntrl) were separately transduced into C2C12 myoblasts before their conversion to quiescence, and after being brought to this state, they were then stimulated with serum (growth medium [GM]) for the indicated times (Fig. 5 A). Remarkably, after performing RT-PCR analysis on myoblasts expressing shMyoD, we observed a severe reduction not only in the levels of *MyoD* mRNA but also in the levels of *Cdc6* mRNA as well (Fig. 5 B). In contrast, quiescent myoblasts, which had been transduced in parallel with either empty vector or shCntrl, had no difficulty in expressing either one of these genes (Fig. 5 B). We also examined the protein levels of Cdc6 after the quiescent myoblasts were serum stimulated as a function of time, and as shown in Fig. 5 C, the depletion of MyoD, either by shMyoD-64 or -66, resulted in the near absence of Cdc6 protein when compared with shCntrl. As expected, the protein profiles of E2F3a and Myf5 were found to be unaffected in the MyoD-depleted myoblasts expressing shMyoD-66 (Fig. S2 E and not depicted).

Next, we performed ChIP analysis on serum-stimulated quiescent myoblasts expressing either shMyoD-66 or the scrambled shRNA to examine the occupancy of the *Cdc6* promoter under conditions of MyoD ablation. Fig. 5 D clearly demonstrates that both MyoD and E2F3a were recruited to the promoter of *Cdc6* after quiescent myoblasts expressing the scrambled shRNA were stimulated with serum for 6 h. In sharp contrast, we were unable to detect any significant binding of MyoD or E2F3a to the promoter of *Cdc6* after the quiescent myoblasts expressing shMyoD-66 were stimulated with serum for periods of 6, 12, or 16 h (Fig. 5 E and not depicted). This result implies that E2F3a may require MyoD for its recruitment to the *Cdc6* promoter after quiescent satellite cells progress into the cell cycle.

A

shRNA → 0 h
(-Met) → 48 h
GM → 84 h

Harvest 0 h   16 or 36 h

B

	Phase	BrdU (0 h)	Phase	BrdU (16 h)	Phase	BrdU (36 h)
shCntrl						
shMyoD-66						

BrdU(+)/DAPI(+) %

shCntrl
shMyoD-66

Figure 6. **Depletion of MyoD in growth-stimulated quiescent myoblasts is associated with a reduction in the number of cells entering S phase.** (A) C2C12 myoblasts were transduced with lentiviruses (shMyoD-66 or shRNA control), and after 48 h, they were brought to quiescence, at which time serum (GM) was then added for either 16 or 36 h. (B) To monitor DNA synthesis, BrdU was added to the media for 30 min at each of the respective times, and then immunostained, accordingly, for the detection of BrdU incorporation. The bottom panel is the quantitation of the results, and the bars in the histogram represent the mean percentages of cells positive for BrdU per field of view. Error bars represent SEM. (*, $P < 0.001$). Bar, 20 µm.

Entry of quiescent myoblasts into S phase requires MyoD

Because Cdc6 is such a key factor in preparing chromatin for DNA replication in eukaryotic systems (Borlado and Méndez, 2008), we wanted to determine whether the depletion of MyoD had any effect on the ability of growth-stimulated quiescent myoblasts to enter into S phase, as judged by BrdU incorporation. Thus, by using the strategy described in Fig. 6 A, we found that the percentage of MyoD-depleted myoblasts entering S phase after 16 h of serum stimulation was remarkably reduced when compared with myoblasts with the shCntrl vector (Fig. 6 B). Therefore, we conclude that MyoD is required for inducing Cdc6 in growth-stimulated quiescent myoblasts, and by extrapolation, this activity could signify one mechanism for preparing recently activated satellite cells to enter S phase.
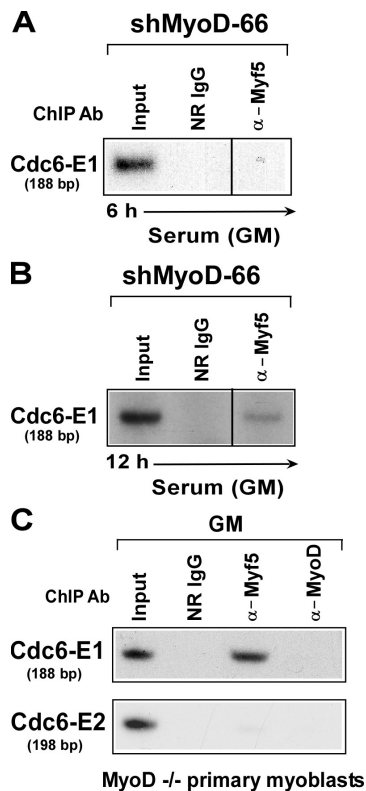
Interestingly, although the percentage of control myoblasts (shCntrl) in S phase after 36 h was virtually identical to that which was seen after 16 h, the percentage of MyoD-depleted myoblasts in S phase at the 36-h time point had increased at least threefold over that which was seen at the l6-h time point (Fig. 6 B). We propose that *Cdc6* may be associated with this increase, only because its expression is unpredictably observed in the MyoD-depleted quiescent myoblasts after 16 h of serum stimulation (Fig. 5 C). Given that this time point shows no evidence for the expression of MyoD but that it does for Myf5 (Fig. 5 C and Fig. S2 E), we thought that *Myf5* might perhaps be responsible for contributing to the expression of *Cdc6* while under these conditions. Therefore, we tested this idea by examining the *Cdc6* promoter for the occupancy of Myf5 in the MyoD-depleted quiescent myoblasts after serum stimulation. In accordance with the pattern of Myf5's expression in growth-stimulated quiescent myoblasts (Fig. S2, C and E), we were unable to detect this protein at the promoter of *Cdc6* after 6 h of serum stimulation (Fig. 7 A) but did detect it after a period of 12 h (Fig. 7 B). Perhaps more striking is that Myf5 can also readily associate with the *Cdc6* promoter in MyoD$^{-/-}$ primary myoblasts, as demonstrated by ChIP analysis (Fig. 7 C). Collectively, these results suggest but do not prove that Myf5 can act as a replacement factor for MyoD in inducing the promoter of *Cdc6*, but only under a given set of circumstances.

Discussion

In summary, the results presented in this study reveal a new and important role for MyoD: it can participate with other transcription factors (e.g., E2F3a) in regulating *Cdc6*, a gene which is extremely important in conferring replication competency to cells that have transitioned out of quiescence (Pelizon, 2003). This surprising observation may be one reason why satellite stem cells express high levels of MyoD within hours after their activation and before muscle-specific gene products are detected (Jones et al., 2005). Indeed, our data clearly demonstrate that when MyoD is ablated in growth-stimulated quiescent myoblasts, Cdc6 is not sufficiently induced, and consequently, these cells cannot fully progress into S phase. Thus, the ability of MyoD to affect full transcription from the *Cdc6* promoter, as shown herein, is a mechanism that more than likely sets the stage for S phase in early activated satellite stem cells. Moreover, our discovery that MyoD can associate with the promoters of other genes (e.g., *MCM2* and *-7*) that are also central to the regulation of DNA leads us to suspect that this activity is associated with parallel mechanisms for promoting these cells into S phase. Finally, our evidence indicating that Myf5 can also occupy and stimulate the *Cdc6* promoter is intriguing from the standpoint that this could reflect a failsafe mechanism for

A shMyoD-66

ChIP Ab — Input | NR IgG | α – Myf5

Cdc6-E1
(188 bp)

6 h ⟶
Serum (GM)

B shMyoD-66

ChIP Ab — Input | NR IgG | α – Myf5

Cdc6-E1
(188 bp)

12 h ⟶
Serum (GM)

C GM

ChIP Ab — Input | NR IgG | α – Myf5 | α – MyoD

Cdc6-E1
(188 bp)

Cdc6-E2
(198 bp)

MyoD -/- primary myoblasts

Figure 7. **Myf5 can occupy the promoter of *Cdc6* in MyoD$^{-/-}$ primary myoblasts and in MyoD-depleted quiescent myoblasts after serum stimulation.** (A and B) Cross-linked chromatin prepared from quiescent C2C12 myoblasts expressing shMyoD-66 after stimulation by serum for periods of 6 or 12 h was immunoprecipitated in parallel with anti-Myf5 and normal rabbit IgG (NR IgG). Semiquantitative PCR was then performed with the use of primers surrounding the E-box site E1 within the *Cdc6* promoter. Black lines indicate that intervening lanes have been spliced out. (C) Crossed-linked chromatin from cultured MyoD$^{-/-}$ primary myoblasts was immunoprecipitated in parallel with anti-Myf5 or anti-MyoD antibody (Ab) and then analyzed by semiquantitative PCR using primers surrounding the E-box sites (E1 and E2) within the *Cdc6* promoter. A control reaction with the use of normal rabbit IgG is shown along with input DNA, which was amplified by the same set of primers.

assuring that activated satellite cells progress into S phase under conditions of MyoD impairment. This prediction is strengthened by the fact that ~80% of the quiescent satellite cells, which are committed for myogenesis, transcriptionally express Myf5 (Buckingham, 2007), and exactly what function this provides in this context is still unclear. Therefore, a more general understanding of the roles of both MyoD and Myf5 during the proliferative expansion of adult myogenic stem cells is important from the perspective of new therapeutic approaches for neuromuscular diseases.

Materials and methods

Single myofiber isolation and culturing
Male C57BL/6 wild-type mice of 6 wk old (The Jackson Laboratory) were used in this study and with the approval of the Institutional Animal Care and Use Committee at Case Western Reserve University. The extensor digitorum longus muscles were removed from mice after being killed by cervical dislocation (Rosenblatt et al., 1995). To minimize satellite activation, muscles were digested without shaking in a dish with 1× DME plus 0.2% collagenase type 1 for 45 min at 35°C, as previously described (Rosenblatt

et al., 1995; Wozniak and Anderson, 2005; Parise et al., 2008). Individual myofibers were dissociated by trituration, washed, and then cultured in a horse serum (HyClone)–coated Petri dish (Kord-Valmark) with added DME to maintain the associated satellite cells in a quiescent state. To activate the associated satellite cells, the cultures were switched to DME and 20% FBS with 0.5% chick embryo extract (Sera Laboratories International, Ltd) and then incubated at 37°C in 5% CO$_2$ until harvesting. At least three to four mice per time point were used.

Cell culture and quiescence
C3H10T1/2 mouse fibroblasts and 293T cells were maintained in DME supplemented with antibiotics and 10% FBS. C2C12 skeletal muscle cells (provided by N. Rosenthal, European Molecular Biology Laboratory Monterotondo, Rome, Italy) were maintained in DME supplemented with 20% FBS (GM) as previously described (Mal et al., 2000, 2001). To prepare myotubes, myoblasts were switched to differentiating medium (DM; DME, 2% heat-inactivated horse serum, and 10 µg/ml insulin), and after 12 h, 10 µM cytosine arabinoside was added for 36 h to eliminate proliferating, nondifferentiating myoblasts (Mal et al., 2000). Methods for removing myotubes from culture dishes without quiescent undifferentiated myoblasts were as previously described (Kitzmann et al., 1998). In brief, trypsin at a concentration of 0.05% was added to cultures of myotubes followed by incubation at 37°C for ~20–30 min.

Except for minor modifications, C2C12 myoblasts were made quiescent as previously described (Kitzmann et al., 1998; Spiller et al., 2002). In brief, myoblasts were grown on tissue culture–treated polystyrene dishes (Corning) and in medium consisting of high-glucose DME (Invitrogen) supplemented with heat-inactivated 20% FBS. Upon reaching 40–50% confluency, the cells were then switched after extensive washings in warm PBS to high-glucose DME without methionine and with 1% FBS (quiescent medium) for 36 or 48 h. Quiescent myoblasts, whose confluency was ~30–40%, were promoted into the cell cycle by changing the medium to fresh DME with 20% FBS (GM).

shRNA-mediated gene silencing
Lentiviral-based shRNA constructs (pLKO.1) expressing shRNAs targeting the sequences of nucleotides 1,061–1,081, 1,044–1,064, or 1,126–1,146 of MyoD (GenBank/EMBL/DDBJ accession no. NM_010866) were purchased from Thermo Fisher Scientific and renamed shMyoD-63, -64, and -66, respectively. A pLKO.1 construct expressing a scrambled shRNA was purchased from Addgene and served as a negative control (shCntrl). To produce lentivirus, each of the pLKO.1 constructs was cotransfected with psPAX2 and pMD2G into 293T packaging cells as previously described (Jackson et al., 2005). Supernatants derived from these cells were then used to infect C2C12 myoblasts.

Promoter sequence analysis, expression plasmids, and gene reporter assays
Putative binding sites for MyoD (E-boxes) and E2F were identified along the 1.3-kb *Cdc6* promoter by analysis with the TRANSFAC (version 7.0) computer software program (Matys et al., 2006). The *Cdc6* promoter fragment was generated by PCR from mouse genomic DNA, ligated into the pGL3-basic luciferase reporter vector (Promega), and then sequenced accordingly. PCR primers (forward, 5′-AGACCTGGGGCTGTCCTATT-3′; and reverse, 5′-CTGCTCAAAACTAGCCAGCA-3′) were designed to amplify 449 bp (−168, +281) of the *Cdc6* promoter sequence (GenBank/EMBL/DDBJ accession no. NM_001025779), which is numbered relative to the transcription start site at +1, is described in the NCBI UniGene database (Genome View). Nucleotide substitutions were introduced into the putative E-box or E2F site of Cdc6-wt at position −28 (TGCAGCTGCT converted to TGCAGCAACT) or −48 (TTTGGCGC converted to TTTGAAGC), respectively, by using a site-directed mutagenesis kit (Agilent Technologies). Nucleotide substitutions were also introduced into both of these sites to generate a double mutant. The DNA sequences of all of these constructs were verified accordingly.

The plasmid pCSA-MyoD expressing full-length MyoD from the CMV promoter (pCSA-MyoD) has been described previously (Skapek et al., 1996), as have the CMV promoter–driven plasmids pHIT3-Myf5 and pcDNA-mE2F3a (Wu et al., 2001; Parker et al., 2006), both of which were provided by M.A. Rudnicki (Ottawa Health Research Institute, Ottawa, Ontario K1Y 4E9, Canada) and G. Leone (The Ohio State University, Columbus, OH), respectively.

Luciferase assays were carried out by using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instruction. To control for transfection efficiency, all firefly luciferase values were normalized to Renilla luciferase.

Transfections and monitoring of DNA synthesis

For reporter gene assays, cultures of C3H10T1/2 or C2C12 myoblasts were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Primary mouse wild-type and MyoD$^{-/-}$ myoblasts (provided by M.A. Rudnicki) were isolated and cultured as previously described (Sabourin et al., 1999).

To monitor for DNA synthesis, a BrdU Labeling and Detection kit (Roche) was used. In brief, C2C12 myoblasts cultured in GM, DM, or quiescent medium were labeled with 10 µM BrdU for 30 min and then processed according to the manufacturer's instructions. The secondary antibody for BrdU staining was anti–mouse Alexa Fluor 568 or 488.

Antibodies, immunoblotting, and immunofluorescence

Antibodies recognizing MyoD (C-20 and M-318), Myf5 (C-20), E2F3a (C-20), Cdc6 (180.2 and H-304), E2F4 (C-20), E2F1 (KH95), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 6C5), and normal rabbit IgG were obtained from Santa Cruz Biotechnology, Inc. Hybridoma cells producing the anti-Pax7 mouse monoclonal antibody were provided by M.A. Rudnicki, and Alexa Fluor 594– or Alexa Fluor 488–conjugated goat anti–mouse IgG secondary antibodies for detecting primary antibodies were purchased from Invitrogen. The spectral compatibility for these two fluorophores can be seen at http://www.invitrogen.com.

Lysis of C2C12 myoblasts and conditions for immunoblot analysis were described previously (Mal et al., 2001). In brief, 20–40 µg of extracts was separated on 10% SDS-PAGE followed by transfer to polyvinylidene difluoride membranes, which were then probed with the primary antibody and, afterward, the secondary antibody followed by ECL (GE Healthcare). Immunofluorescence was performed on myoblasts or myotubes as previously described (Mal et al., 2000, 2001).

Myofibers were fixed at the various time points indicated in Results and then immunostained according to previously published procedures (Kuang et al., 2006). In brief, fixative solution consisting of 4% paraformaldehyde-PBS was added to the myofibers for 10 min at RT and then washed with 100 mM glycine-PBS to stop the fixation. Afterward, the myofibers were washed three times with PBS, placed into blocking solution (0.2% Triton X-100–PBS with 1% BSA, 0.1% Azide, and 5% goat serum) containing the primary antibody, and then stored overnight at 4°C. After this, the myofibers were washed three times in PBS and then incubated with the appropriate secondary antibody at a dilution of 1:3,500 or 1:1,000 for Alexa Fluor 594 and 488, respectively.

Microscope and image capture

Fluorescence and phase-contrast images of cells or myofibers in PBS were acquired using an inverted microscope (DMI 4000B; Leica) with accompanying dye set filters and 20× NA 0.40 or 40× NA 0.55 objectives at RT. Images were digitally captured using a cooled color 12-bit camera (Retiga 2000R Fast 1394; QImaging) with QCapture Pro software (version 6.0; QImaging). Images were optimized for color balance, brightness, and contrast using Photoshop CS3 (Adobe Systems, Inc.) and then assembled into figures using Canvas X (ACD Systems, Inc.).

ChIP

ChIP analysis was performed as previously described (Parker et al., 2006) but with modifications. Primary mouse myoblasts, C2C12 myoblasts, or myotubes were cross-linked with 1% formaldehyde for 10 min, and afterward, the cells were harvested, washed with cold PBS, and resuspended in nuclear SDS lysis buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1, and 1× Complete antiprotease cocktail (Roche). The lysate was then sonicated by Bioruptor (Diagenode Inc.) under conditions that consistently yielded DNA fragment sizes of 100–400 bp. Sonicated chromatin was clarified and then diluted 10-fold in immunoprecipitation buffer (16.7 mM Tris, pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, and 0.01% SDS) to bring the final concentration of SDS to 0.1%. ChIP reactions were performed overnight at 4°C with 50 µg chromatin and 1–2 µg of specific antibodies. Afterward, the immunocomplexes were extensively washed, accordingly, and reverse cross-linked at 65°C overnight. DNA was recovered by phenol/chloroform, ethanol precipitated, and then quantitated by using a fluorospectrometer (ND-3300; NanoDrop Technologies). The DNA precipitates and input (0.05% of total chromatin used for the immunoprecipitation) were analyzed by semiquantitative PCR using corresponding primers for the indicated promoter. The sequences used were Cdc6-E1 (forward, 5′-TGATGAGTGACAACTAATCAG-3′; and reverse, 5′-GAGCTTTGCACTCTTCAGG-3′), Cdc6-E2 (forward, 5′-AGG-TAACTGGCGATTGTGCT-3′; and reverse, 5′-GCTCTGGCTGTCTTG-GAACT-3′), and Mcm2 (forward, 5′-CCTTCCGCTCTAGATTGACG-3′; and reverse, 5′-AGAGGAAGATACCGGCCAAG-3′). PCR was performed by

using a PCR Master Mix kit (Roche) in a reaction volume of 25 µl consisting of 3.0 µl of precipitated DNA, 10 pmol of the appropriate primers, and 0.1 µCi α-[^{32}P]dCTP in buffer. The thermal cycle program for amplification of the *Cdc6* promoter sequence was 94°C for 3 min, followed by 29 cycles of 94°C for 15 s, 54°C for 30 s, and then 72°C for 30 s. PCR products were resolved on 8% native polyacrylamide gels and visualized by autoradiography.

RT-PCR

Total RNA was prepared using the TRIZOL reagent (Invitrogen) accordingly. cDNA was generated by using the First-Strand cDNA Synthesis kit (GE Healthcare), and this was then amplified by PCR using specific primers spanning at least two exon regions of Cdc6, MyoD, or GAPDH. Quantification of GAPDH levels in the same cDNA samples measured in separate PCR reactions served as an endogenous control. Each product was resolved by agarose gel electrophoresis and visualized by ethidium bromide staining.

Online supplemental material

Fig. S1 shows that MyoD can occupy the *Mcm2* promoter in myoblasts, and reporter assays show that the regulatory region of *Cdc6* is responsive to MyoD, E2F3a, or Myf5 in C3H10T1/2 cells. Fig. S2 shows the conversion of C2C12 myoblasts to a quiescent state, the analysis of Myf5 and E2F3a expression in serum-stimulated quiescent myoblasts, the colocalization of MyoD and Cdc6 in activated satellite cells in association with myofibers, and the expression of Myf5 in quiescent myoblasts depleted of MyoD after serum stimulation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904144/DC1.

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