B*2705 was not due to a quantitative effect, because peptide 4 generated a more stable conformation of B*2705 molecules (Fig. 3A) yet did not restore protection from any NK clone (Fig. 3B). The protective effect of peptide 1 required B*2705 expression: Untransfected RMA-S and T2 cells incubated with peptide 1 were still lysed by NK cells (Fig. 4). Data obtained with T2-B27 cells confirmed the protective effect of peptide 1 with clones 2wA-29 and 2w-14/C1R (Fig. 4).

The requirement for a specific peptide in the formation of a protective structure virtually eliminates the possibility that NK cells are triggered by a target structure bound to class I and released by peptide binding, as postulated by the masking hypothesis (*2*). The peptide specificity in class I recognition by NK cells can explain the heterogeneity among NK clones in their ability to lyse virus-infected targets (*24*): The loss of protection from NK lysis caused by virus infection no longer implies replacement of most endogenous peptides but could occur through interference with the formation of specific class I–peptide complexes.

Our data show that a fully assembled class I molecule is competent for an effective interaction with NK receptors, as measured by the protection of target cells from NK-mediated lysis, and that peptides contribute directly to such protective conformations. In addition, NK cells exhibit peptide specificity, as suggested by the distinct sensitivity of different NK clones to single amino acid changes in the peptide binding site and as demonstrated by the ability of two NK clones to discriminate among four peptide-dependent configurations of the same B*2705 molecule. These results also imply the existence of multiple NK receptors able to distinguish among different class I–peptide complexes.

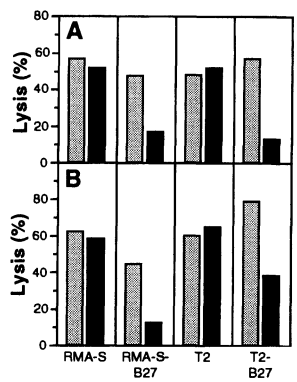

Fig. 4. The protection from lysis provided by peptide 1 is dependent on B*2705 expression, both in RMA-S and in T2 cells. (**A**) Lysis by NK clone 2wA-29 (effector:target ratio of 0.5) of the indicated cells incubated without (shaded bars) or with peptide 1 (filled bars) as in Fig. 3. (**B**) Lysis by NK clone 2w-14/C1R as in (A).

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10. $CD3^-$ NK clones were generated from normal donors that lacked B*2705, B*0801, and B*1402 alleles. Clones were characterized as described (*24*). Lysis of target cells was determined by a standard 3-hour chromium-release assay (*24*).
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15. The IgM mAb A6/136 was isolated on the basis of its ability to abrogate the class I–mediated protection of target from lysis by NK cells (*25*). The IgM B27-specific mAb B27.2M and the IgG1 B27–specific mAb ME.1 were obtained from American Type Culture Collection, Rockville, MD.
16. E45T indicates the replacement of the glutamic acid residue 45 by threonine. L, leucine; I, isoleucine; D, aspartic acid; and F, phenylalanine. A more extensive study with a larger number of NK clones and additional mutants of B*2705 will be reported elsewhere.
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26. Peptides 1 to 3 correspond to peptides naturally bound to B*2705 (*17*). Peptide 1 (FRYNGLIHR), peptide 2 (RRISGVDRY), peptide 3 (ARLFGIRAK), and peptide 4 (GRFGGGGGL) have been used to form complexes in vitro with soluble B*2705 molecules and β_2-microglobulin (*18*).

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Correlation of Terminal Cell Cycle Arrest of Skeletal Muscle with Induction of p21 by MyoD

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Skeletal muscle differentiation entails the coordination of muscle-specific gene expression and terminal withdrawal from the cell cycle. This cell cycle arrest in the G_0 phase requires the retinoblastoma tumor suppressor protein (Rb). The function of Rb is negatively regulated by cyclin-dependent kinases (Cdks), which are controlled by Cdk inhibitors. Expression of MyoD, a skeletal muscle–specific transcriptional regulator, activated the expression of the Cdk inhibitor p21 during differentiation of murine myocytes and in nonmyogenic cells. MyoD-mediated induction of p21 did not require the tumor suppressor protein p53 and correlated with cell cycle withdrawal. Thus, MyoD may induce terminal cell cycle arrest during skeletal muscle differentiation by increasing the expression of p21.

Myogenic basic helix-loop-helix (bHLH) proteins, such as MyoD, promote skeletal muscle–specific gene expression and permanent cell cycle arrest (*1*). Forced expression of MyoD can inhibit cell cycle progression independently of muscle differentiation (*2*), but the molecular basis for this effect and its relation to differentiation of normal skeletal muscle are not known. Members of the retinoblastoma family of tumor suppressor proteins are important for myogenic differentiation. Their inactivation by viral oncoproteins both blocks differentiation and induces DNA synthesis in otherwise terminally differentiated myocytes [reviewed in (*3*)]. A specific role for Rb has been demonstrated as differentiated skeletal muscle cells express large amounts of hypophosphorylated (active) Rb (*4*, *5*), and specific loss of Rb through gene inactivation pre-

Table 1. Induction of p21 by MyoD in CV1 and U2OS cells correlates with cell cycle arrest. Both cell lines were transiently transfected with CMV-MyoD (pCSA-MyoD) and maintained in GM. The induction of p21 was monitored by simultaneous immunostaining for MyoD and p21 (Fig. 3C). DNA synthesis was evaluated by measuring the uptake of the thymidine analog BrdU over a 24-hour period in a parallel dish of each cell type stained for MyoD (*31*). At least 300 cells were counted in each category, and results are representative of multiple experiments performed.

Cell type	MyoD$^+$ cells		MyoD$^-$ cells	
	p21$^+$ (%)	BrdU$^-$ (%)	p21$^+$ (%)	BrdU$^-$ (%)
CV1	46.7	50.3	1.5	1.9
U2OS	13.0	14.5	5.2	3.2

vents the normal G_0 arrest observed in differentiated skeletal muscle (*6*, *7*).

Because Rb is essential in maintaining the postmitotic state of differentiated myocytes, we sought to determine the mechanism that ensures that Rb remains functionally active in these cells. Rb family members are functionally regulated by Cdks and their regulatory partners, the cyclins (*8*). One mechanism by which these molecules regulate the transition from the G_1 phase to the S phase of the cell cycle is phosphorylation of Rb, which relieves repression of cellular transcription factor complexes such as E2F-DP resulting in activation of genes important for the initiation of DNA synthesis (*9*). Molecules that inhibit the activity of Cdks have been identified: p21 (also known as WAF1, CIP1, CAP20, sdi1, and Pic1) (*10*–*12*), p27 (also known as p27^{Kip1}) (*13*, *14*), p16 (also known as p16^{INK4}) (*15*), and p15 (also known as p15^{INK4B}) (*16*). Because Cdk inhibitors inactivate negative regulators of Rb, these molecules may positively regulate Rb function. Indeed, overexpression of p21 or p27 induces cell cycle arrest (*10*, *11*, *14*), as does overexpression of Rb (*17*). Therefore, we hypothesized that myogenic factors might indirectly regulate Rb function by inducing the expression of Cdk inhibitory proteins.

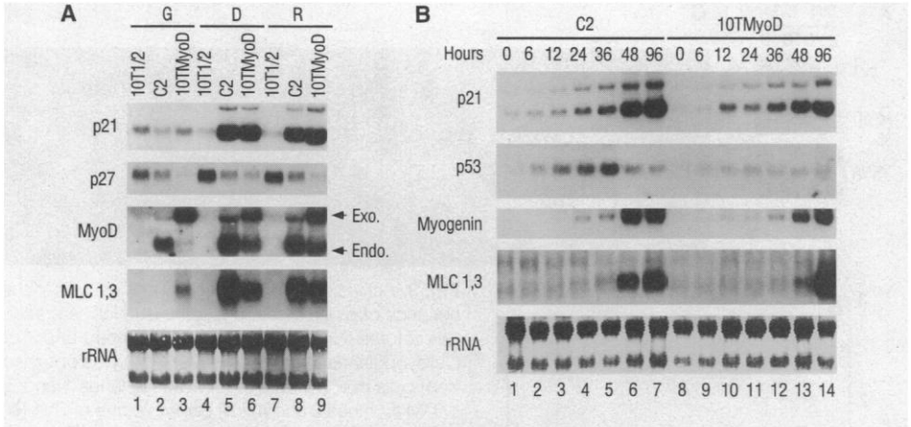

Fig. 1. Expression of p21 induced by MyoD during muscle differentiation. (**A**) Northern (RNA) analysis of total RNA isolated from 10T1/2 fibroblasts (lanes 1, 4, and 7), C2 myoblasts (lanes 2, 5, and 8), and 10TMyoD cells (10T1/2 cells expressing retroviral MyoD; lanes 3, 6, and 9) (*29*). RNA was isolated from proliferating cells maintained in GM (G, lanes 1 to 3), confluent cells maintained for 96 hours in DM (D, lanes 4 to 6), or cells that had been maintained in DM for 96 hours and then in GM for 24 hours (R, lanes 7 to 9). Amounts of p21, p27, MyoD, and myosin light chain (MLC 1,3) RNA were determined. Viral (Exo.) and cellular (Endo.) transcripts encoding MyoD are indicated. (**B**) Time course of p21 induction. RNA was obtained from C2 (lanes 1 to 7) or 10TMyoD myoblasts (lanes 8 to 14) incubated in DM for the times indicated. Amounts of p21, p53, myogenin, and myosin light chain (MLC 1,3) RNA were determined by Northern analysis. (**C**) Protein immunoblotting of p21 and p27 from 10T1/2, 10TMyoD, and C2 cells cultured as in (A) (*29*).

Expression of both p21 RNA and protein was induced during differentiation of C2 muscle cells (Fig. 1, A and C). In contrast, p21 RNA and protein amounts decreased in 10T1/2 fibroblasts that were incubated under the same low-serum conditions used to induce muscle differentiation (differentiation medium, DM). However, when 10T1/2 cells were stably infected with a retrovirus encoding MyoD (10TMyoD cells) and incubated in DM, p21 expression was then induced (Fig. 1, A and C). In both C2 and 10TMyoD myoblasts, induction of p21 RNA was detectable after 12 hours in DM and steadily increased up to 96 hours (Fig. 1B). The increase in the amount of p21 RNA slightly preceded the expression of muscle differentiation markers (myogenin and myosin light chain), suggesting that p21 may be directly induced by MyoD. Because differentiated myotubes do not re-enter the cell cycle in response to mitogen stimulation, we determined whether p21 expression persisted under these conditions. Indeed, enhanced expression of p21 was maintained in differentiated muscle cells after re-addition of

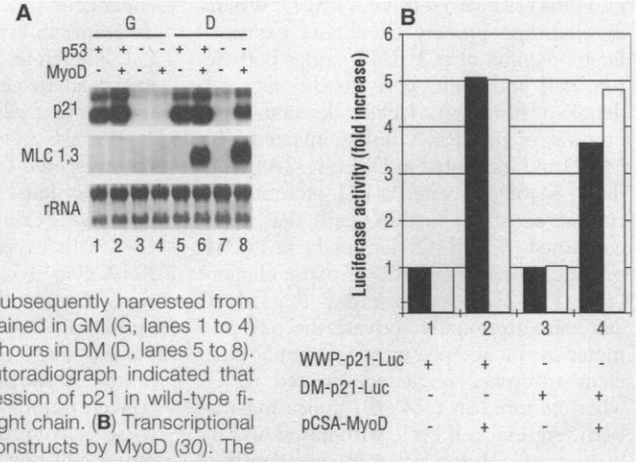

Fig. 2. Induction of p21 by MyoD in the absence of p53. (**A**) Northern analysis of p21 expression in wild-type (lanes 1, 2, 5, and 6) and p53-deficient (lanes 3, 4, 7, and 8) mouse primary embryonic fibroblasts (*29*). Subconfluent fibroblasts were either mock-infected (odd lanes) or infected with an ecotropic MyoD retrovirus (even lanes) (*21*). RNA was subsequently harvested from either proliferating cells maintained in GM (G, lanes 1 to 4) or from cells incubated for 48 hours in DM (D, lanes 5 to 8). Shorter exposures of the autoradiograph indicated that MyoD similarly induced expression of p21 in wild-type fibroblasts. MLC 1,3; myosin light chain. (**B**) Transcriptional activation of p21 promoter constructs by MyoD (*30*). The p53-deficient fibroblasts were transfected with WWP-p21-Luc or DM-p21-Luc (*10*) and either an empty CMV expression vehicle (pCSA) (lanes 1 and 3) or CMV-MyoD (pCSA-MyoD) (lanes 2 and 4). Luciferase activity is expressed as the fold increase over that achieved in the absence of MyoD cotransfection. Duplicate samples (open and closed bars) from a representative experiment are displayed.

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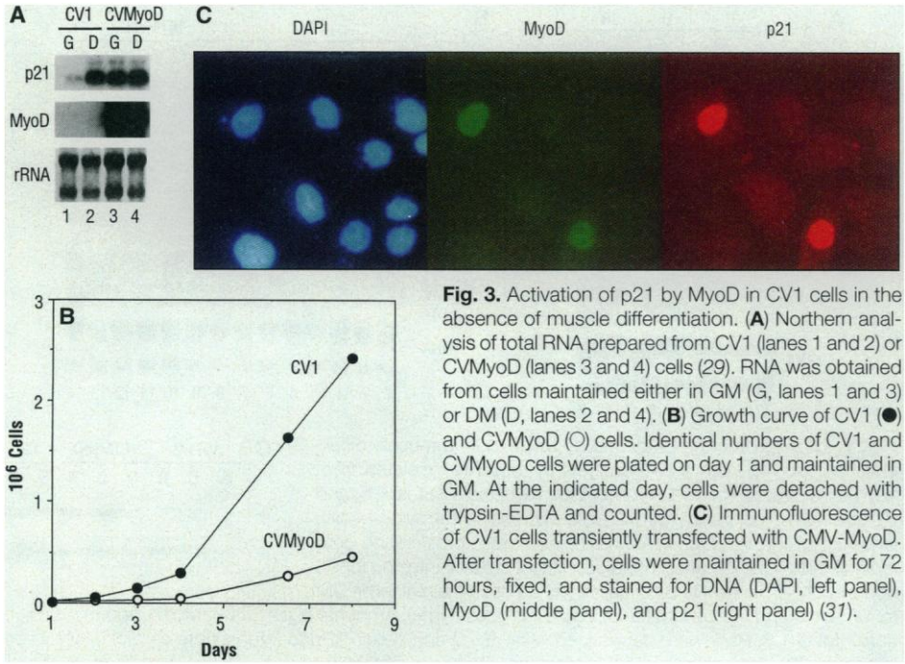

Fig. 3. Activation of p21 by MyoD in CV1 cells in the absence of muscle differentiation. (**A**) Northern analysis of total RNA prepared from CV1 (lanes 1 and 2) or CVMyoD (lanes 3 and 4) cells (*29*). RNA was obtained from cells maintained either in GM (G, lanes 1 and 3) or DM (D, lanes 2 and 4). (**B**) Growth curve of CV1 (●) and CVMyoD (○) cells. Identical numbers of CV1 and CVMyoD cells were plated on day 1 and maintained in GM. At the indicated day, cells were detached with trypsin-EDTA and counted. (**C**) Immunofluorescence of CV1 cells transiently transfected with CMV-MyoD. After transfection, cells were maintained in GM for 72 hours, fixed, and stained for DNA (DAPI, left panel), MyoD (middle panel), and p21 (right panel) (*31*).

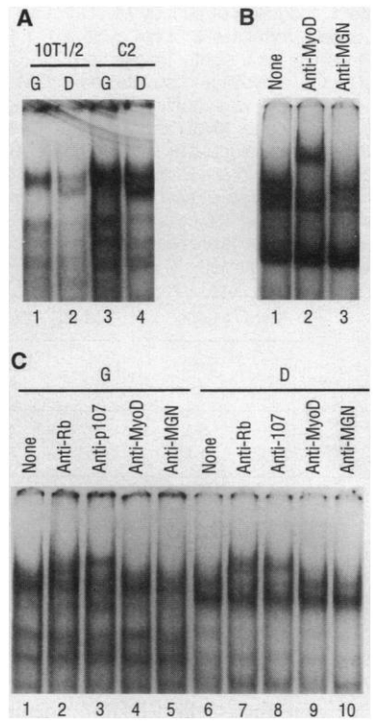

Fig. 4. Absence of myogenic bHLH proteins from E2F complexes in muscle cells. (**A**) Electrophoretic mobility-shift assay (EMSA) of E2F site binding activities in nuclear extracts from 10T1/2 fibroblasts or C2 myoblasts that were maintained either in GM (G, lanes 1 and 3) or DM for 72 hours (D, lanes 2 and 4) (*32*). (**B**) EMSA of MEF1 site binding activities in nuclear extracts from differentiated C2 myotubes. Before gel electrophoresis, extracts were incubated with either buffer alone or the indicated monoclonal antibodies. MGN, myogenin. (**C**) EMSA of E2F binding site activities in nuclear extracts from either proliferating (lanes 1 to 5) or differentiated (lanes 6 to 10) C2 myocytes incubated with antibodies as in (B).

growth medium (GM) (Fig. 1, A and C).

The amount of p27 RNA remained relatively constant in both proliferating and serum-deprived fibroblasts and myogenic cells (Fig. 1A). However, the amount of p27 protein increased in confluent cultures of both fibroblasts and myogenic cells incubated in DM, and the amount remained elevated in these cultures after addition of GM (Fig. 1C). Thus, the amount of p27 protein seems to be posttranscriptionally modulated, perhaps by cell-cell contact, independently of the muscle differentiation program.

Because p21 is a p53-inducible gene (*10, 18*), we determined whether MyoD-mediated p21 expression requires p53 by infecting p53-deficient mouse primary embryonic fibroblasts (*19*) with retroviral MyoD. Whereas wild-type primary fibroblasts expressed large amounts of p21 RNA under both serum-rich and serum-poor conditions, p53-deficient fibroblasts lacked detectable expression of p21 RNA unless infected with MyoD and incubated in DM (Fig. 2A). Similarly, MyoD activated a p21 promoter–luciferase construct in these cells that either contained (WWP-p21-luc) or lacked (DM-p21-luc) an upstream p53 response element (*10*) (Fig. 2B), demonstrating that MyoD can transcriptionally activate the p21 promoter in the absence of p53. The p53-deficient myotubes remained arrested in G_0 when cultured in GM (*6*), indicating that both terminal cell cycle withdrawal and induction of p21 can occur independently of p53 in skeletal muscle. However, p53 RNA expression was transiently increased during muscle differentiation (Fig. 1B) (*20*) and may act synergistically with MyoD to activate p21 expression in wild-type cells.

MyoD can induce cell cycle arrest in some cell types, such as CV1 cells, that cannot be induced to express any markers of muscle differentiation (*21*), suggesting that MyoD-induced cell cycle arrest can be uncoupled from muscle differentiation (*2*). To see whether induction of p21 correlates with the ability of MyoD to induce cell cycle arrest in the absence of muscle differentiation, we transiently transfected CV1 cells with a MyoD expression plasmid (CMV-MyoD). Approximately the same proportion of MyoD-positive CV1 cells that expressed p21 (Fig. 3C) also failed to synthesize new DNA (Table 1). This correlation was also observed in human osteosarcoma cells (U2OS) that can be induced to differentiate into skeletal muscle by MyoD (*5*). Therefore, forced expression of MyoD may lead to cell cycle arrest through the induction of p21.

We also monitored p21 expression in a nonmyogenic line of CV1 cells that stably expresses large amounts of ectopic MyoD (CVMyoD cells). Proliferating parental CV1 cells expressed small amounts of p21 RNA (Fig. 3A) and protein (*6*) which increased when these cells were deprived of serum. In contrast, CVMyoD cells expressed large amounts of p21 RNA and protein in the presence of either high or low concentrations of serum. Accordingly, these cells grew more slowly than the parental cell line (Fig. 3B). Whereas MyoD-mediated induction of p21 in muscle cells is restricted to differentiated cells, MyoD can apparently induce p21 amounts in CV1 cells maintained in GM that are compatible with continued cell proliferation.

It has been suggested that MyoD promotes cell cycle arrest by directly binding to and affecting the function of Rb (*5, 7*), which mediates cell cycle arrest by interacting with E2F-DP complexes (*9*). Thus, if a direct MyoD-Rb interaction plays a role in cell cycle withdrawal, myogenic bHLH factors may be associated with Rb-E2F complexes in differentiated skeletal muscle. Proliferating fibroblasts and myoblasts contained several E2F binding activities, one of which was a prominent slowly migrating species (Fig. 4A). In both serum-deprived fibroblasts and differentiated myotubes, the faster migrating E2F binding activities disappeared, and a slowly migrating species that had not been present in proliferating cells accumulated. Antibodies to Rb or p107 supershifted the most slowly migrating E2F binding species from both proliferating myoblasts and differentiated myotubes (Fig. 4C). The prominent faster migrating E2F binding spe-

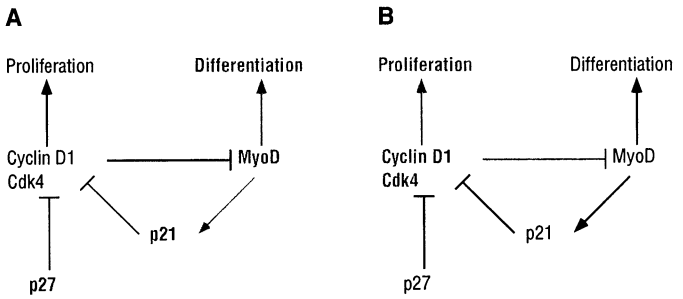

Fig. 5. A positive feedback loop maintains permanent cell cycle withdrawal of differentiated myocytes. (**A**) Active cyclin D1–Cdk4 complexes both drive the cell cycle and inhibit the activity of MyoD in proliferating myoblasts. (**B**) After muscle differentiation is initiated, MyoD activates the expression of p21 which, together with other Cdk inhibitors such as p27, inhibits Cdk activity and thereby maintains cell cycle arrest and ensures that MyoD remains active. In these models, active regulators are shaded black and inactive (suppressed or below threshold) regulators are shaded gray.

cies that was induced in both serum-deprived fibroblasts and differentiated myotubes did not contain Rb or p107, but was specifically supershifted by an antibody to p130 (*22*). Antibodies to MyoD or myogenin did not alter the electrophoretic mobility of E2F complexes from either myoblasts or myotubes (Fig. 4C), though they either supershifted or eliminated protein-DNA complexes containing a MEF1 site oligomer bound by MyoD or myogenin complexes, respectively (Fig. 4B). The absence of associated myogenic factors in E2F complexes present in muscle cell extracts is consistent with the identical electrophoretic mobility of E2F-Rb, E2F-p107, and E2F-p130 complexes in both fibroblasts and myogenic cells (Fig. 4A). Taken together, these data are consistent with MyoD regulating the activity of Rb through an indirect mechanism (*23*).

Forced expression of cyclin D1 inhibits the ability of MyoD to activate the transcription of muscle-specific genes (*24*, *25*). We have also found that ectopic expression of Cdk inhibitors (p21 and p16) in myoblasts maintained in high serum promotes differentiation (*25*). Therefore, we propose that induction of Cdk inhibitors by either cell-cell contact (p27) or muscle differentiation (p21) activates a positive regulatory loop that ensures that MyoD remains functionally active in differentiated myotubes in the presence of growth factors and that differentiated muscle cells remain terminally withdrawn from the cell cycle (Fig. 5). Indeed, heterokaryon analyses have indicated that differentiated myocytes contain a dominant inhibitor of cell cycle progression (*26*). Induction of p21 has also been observed in the differentiation of intestinal epithelial cells in vivo (*27*) and keratinocytes in vitro (*28*), suggesting that induction of Cdk inhibitors may contribute to either the initiation or maintenance of terminal differentiation in a number of cell types.

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28. P. Dotto, personal communication.
29. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (20%) (growth medium). Cells were differentiated in DMEM containing horse serum (2%) and insulin (10 μg/ml) (differentiation medium) for the times indicated. Northern analysis was performed essentially as described (*20*). Relative amounts and integrity of ribosomal RNA (rRNA) were evaluated by staining nylon membranes with methylene blue. The mouse p21 probe was provided by W. El-Deiry (*10*), and the mouse p27 probe was provided by J. Massagué (*14*). For protein immunoblot analysis, cells were lysed essentially as described by Y. Xiong, H. Zhang, D. Beach, *Genes Dev.* **7**, 1572 (1993). Whole-cell extracts containing equivalent amounts of DNA were resolved on a SDS–15% polyacrylamide gel, and transferred proteins were detected with rabbit antisera to p21 (generated by G.J.H. and D.B.) or p27 (generated by H. Toyoshima and T. Hunter) according to the manufacturer's protocol (ECL, Amersham).
30. For luciferase assays, $p53^{-/-}$ mouse primary embryonic fibroblasts were transfected with Lipofectamine (Gibco BRL) according to the manufacturer's directions with 1.0 μg of a luciferase reporter plasmid and 1.0 μg of either pCSA or pCSA-MyoD per transfection. After 72 hours under differentiation conditions, cells were collected and luciferase activity was quantitated with an enhanced luciferase assay kit (Analytical Luminescence Laboratory) according to the manufacturer's directions.
31. Cells were fixed in paraformaldehyde (2%) in phosphate-buffered saline (PBS), and then permeabilized with Triton X-100 (0.25%) in PBS. Cells were immunostained for MyoD and p21 with rabbit antiserum to MyoD [S. J. Tapscott *et al.*, *Science* **242**, 405 (1988)] and a mouse monoclonal antibody to human p21 (Pharmingen), respectively. MyoD immune complexes were detected with antibodies to rabbit immunoglobulin G (IgG) that were conjugated to fluoroscein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories), p21 immune complexes were detected with biotinylated antibodies to mouse IgG and Texas Red avidin D (both from Vector Laboratories), and DNA was detected with 4′,6′-diamidino-2-phenylindole (DAPI). Cells were stained for MyoD and bromodeoxyuridine (BrdU) by staining for MyoD as described above, followed by additional fixation in paraformaldehyde (2%) in PBS and DNA denaturation in 2 N HCl. We detected BrdU with a mouse antibody to BrdU (G3G4) and the reagents described above.
32. The electrophoretic mobility-shift assay (EMSA) for E2F binding activities was performed as described [L. Cao *et al.*, *Nature* **355**, 176 (1992)], and EMSA for MEF1 binding activities was performed as described [A. B. Lassar *et al.*, *Cell* **66**, 305 (1991)]. For antibody supershift experiments, 2.5 μl of either hybridoma-conditioned medium or buffer was used. The following monoclonal antibodies (mAbs) were used: mAb to MyoD (anti-MyoD 5.8A), anti-myogenin (anti-MGN) (F5D), anti-Rb (XZ133), and anti-p107 (SD15).

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