

# Sequestration of pRb by Cyclin D3 Causes Intranuclear Reorganization of Lamin A/C during Muscle Cell Differentiation

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The A-type lamins that localize in nuclear domains termed lamin speckles are reorganized and antigenically masked specifically during myoblast differentiation. This rearrangement was observed to be linked to the myogenic program as lamin speckles, stained with monoclonal antibody (mAb) LA-2H10, were reorganized in MyoD-transfected fibroblasts induced to transdifferentiate to muscle cells. In C2C12 myoblasts, speckles were reorganized early during differentiation in cyclin D3-expressing cells. Ectopic cyclin D3 induced lamin reorganization in C2C12 myoblasts but not in other cell types. Experiments with adenovirus E1A protein that can bind to and segregate the retinoblastoma protein (pRb) indicated that pRb was essential for the cyclin D3-mediated reorganization of lamin speckles. Cyclin D3-expressing myoblasts displayed site-specific reduction of pRb phosphorylation. Furthermore, disruption of lamin structures by overexpression of lamins inhibited expression of the muscle regulatory factor myogenin. Our results suggest that the reorganization of internal lamins in muscle cells is mediated by key regulators of the muscle differentiation program.

## INTRODUCTION

The lamins are the major components of a network of filaments underlying the inner nuclear membrane termed the nuclear lamina. The lamina is an important determinant of interphase nuclear architecture as it plays an essential role in maintaining the integrity of the nuclear envelope (reviewed by Goldman *et al.*, 2002; Hutchison, 2002) and is required for the spatial organization of nuclear functions such as DNA replication (Meier *et al.*, 1991; Moir *et al.*, 1994, 2000; Spann *et al.*, 1997) and RNA polymerase II transcription (Kumaran *et al.*, 2002; Spann *et al.*, 2002). Two major kinds of lamins are present in most mammalian cells: B-type lamins (B1 and B2), which are found in nearly all somatic cells, and A-type lamins (A and C), which are expressed primarily in differentiated cells.

Mutations in human *LMNA* were first observed to cause autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD; Bonne *et al.*, 1999). A number of other mutations affect cardiac, adipose, bone, and neuronal tissues and also cause premature aging (reviewed by Mounkes *et al.*, 2003). Mice that lack *LMNA* develop muscle wasting similar to human EDMD and die soon after birth (Sullivan *et al.*, 1999). The reason for the selective effect on muscle cells is not clear. It has been suggested that the forces generated during muscle contraction might exacerbate physical damage to muscle cell nuclei (Sullivan *et al.*, 1999) or that lamins might influence gene expression in progenitor cells (Wilson, 2000). An

important possibility that needs to be examined is whether lamins play an active role in regulatory events during muscle differentiation.

Differentiation of myoblasts into myotubes is coordinated by two families of transcription factors, MyoD, which includes MyoD, Myf5, myogenin, and MRF4 (Lassar *et al.*, 1994; Rudnicki and Jaenisch, 1995); and MEF2 (Black and Olson, 1998). The MyoD family of factors has the unique property of being able to initiate the myogenic program, even in nonmuscle cells (Davis *et al.*, 1987). The MEF2 proteins cooperate with MyoD to synergistically activate expression of muscle-specific genes. MyoD and MEF2 functions are modulated by various transcriptional coactivators, prominent among these being the coactivator GRIP-1 (Chen *et al.*, 2000), p300/CBP (Eckner *et al.*, 1996), and PCAF (Puri *et al.*, 1997) as well as mitogen-activated signaling pathways (Wu *et al.*, 2000). The interactions of GRIP-1 with MEF2C and myogenin are essential for muscle-specific gene expression (Chen *et al.*, 2000). Both GRIP-1 and MEF2C accumulate in nuclear foci during muscle differentiation (Chen *et al.*, 2001; Lazaro *et al.*, 2002), but the functional significance of this localization is not clear. Early studies with myoblast cell lines in tissue culture (Halevy *et al.*, 1995; Andrés and Walsh, 1996) have established that myoblasts enter the differentiation pathway asynchronously upon depletion of mitogens and express the transcription factor myogenin, followed by the cdk inhibitor p21. After cell cycle exit, the cells differentiate phenotypically, express contractile genes and finally fuse into multinucleated myotubes. Though the expression of most cyclins is down-regulated during cell cycle arrest, a notable exception is cyclin D3 (Kiess *et al.*, 1995; Rao and Kohtz, 1995; Skapek *et al.*, 1995). Up-regulation of retinoblastoma protein (pRb), p21, and cyclin D3 levels occurs during cell cycle withdrawal (Schneider *et al.*, 1994; Guo *et al.*, 1995; Halevy *et al.*, 1995; Novitch *et al.*, 1996; Cenciarelli *et al.*, 1999). pRb plays an essential role in cell cycle arrest, inhibition of DNA synthesis and activation of muscle-specific

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Abbreviations used: DM, differentiation medium; EDMD, Emery-Dreifuss muscular dystrophy; GM, growth medium; pRb, retinoblastoma protein.

genes (Gu *et al.*, 1993; Schneider *et al.*, 1994; Novitch *et al.*, 1996). In differentiated cells, cyclin D3 sequesters hypophosphorylated pRb into a detergent-resistant multiprotein complex (Cenciarelli *et al.*, 1999).

There is currently considerable interest in understanding how nuclear organization regulates processes in the nucleus (Lamond and Earnshaw, 1998; Misteli and Spector, 1998; Shumaker *et al.*, 2003; Spector, 2003). We have earlier observed a novel structural aspect of the lamina in the form of internal lamin A/C speckles that associate with RNA splicing factor domains or speckles in many cell types, using a mAb to recombinant rat lamin A that has certain unique properties (Jagatheesan *et al.*, 1999). This antibody, mAb LA-2H10, exclusively stains intranuclear speckles in interphase cells without labeling the peripheral lamina and specifically recognizes only lamins A and C in immunoblots of cell lysates. Furthermore, expression of siRNA to lamin A/C in HeLa cells disrupts lamin speckles (B. V. Gurudatta and V.K.P., unpublished data). In a process that is unique to muscle cells, lamin speckles are rearranged early during myoblast differentiation to an antigenically masked diffuse network, while preserving the distribution of splicing factor domains and the peripheral lamina (Muralikrishna *et al.*, 2001). This study was undertaken to identify the regulatory factors involved in the reorganization of lamin speckles. We have observed that lamin reorganization occurs during the MyoD-triggered transdifferentiation of fibroblasts to muscle cells. Ectopic cyclin D3 specifically induces lamin reorganization in myoblasts but not in other cell types, and the presence of pRb is essential for this process. Furthermore, disruption of lamin structures inhibits myogenin expression. Our results indicate that lamin reorganization is controlled by key regulators of the muscle differentiation program.

## MATERIALS AND METHODS

### Plasmid Constructs

All the expression constructs used in this study were expressed from the CMV promoter. Expression vectors for MyoD and p21 were obtained from A. Lassar (Harvard Medical School, Boston, MA). pRC-CMV-cycD3-HA and cycD1-HA were obtained from P. Hinds (Harvard Medical School), and the cyclin D3 K112E mutant was constructed in this laboratory by PCR-based mutagenesis. Expression constructs for E1A and its mutants were obtained from M. Harter (Cleveland Clinic Foundation, OH). An expression vector for hCaMKI was provided by A. Means (Duke University Medical Center, Durham, NC). pRC-CMV-cycA and E were obtained from R. Weinberg (Massachusetts Institute of Technology, Cambridge, MA). pSG5-GRIP-1-HA was provided by M. Stallcup (University of Southern California, Irvine, CA) and pcDNA-MEF2C-myc by E. Olson (University of Texas Southwestern Medical Center, Dallas, TX). pCMVcdk4-HA was obtained from S. Van den Heuvel (Massachusetts General Hospital Cancer Center, Boston, MA). A full-length rat lamin A cDNA clone was obtained by extending an available cDNA missing the segment coding for the amino terminal 25 residues (Hamid *et al.*, 1996). Both cDNAs were cloned into the pEGFP-C3 mammalian expression vector (Clontech, Palo Alto, CA). The His-tagged lamin A construct has been described earlier (Kumaran *et al.*, 2002).

### Cell Culture and DNA Transfection

C2C12 mouse skeletal myoblasts (Yaffe and Saxel, 1977; Blau *et al.*, 1983) were maintained at subconfluent densities in DME supplemented with 20% fetal bovine serum (FBS; growth medium, GM). Myogenic differentiation was induced by changing subconfluent cells to DME containing 2% horse serum (differentiation medium, DM). DNA transfections were carried out with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Cells were processed for immunofluorescence microscopy 24 h after transfection. Transfection efficiencies were 25–30% in C2C12 cells and ~20% in other cell types. HeLa cells, NIH3T3, and C3H10T1/2 fibroblasts were grown in DME containing 10% FBS.

### Antibodies

Antibodies to recombinant rat lamins used in this study and characterized in detail previously are mAb LA-2H10, that recognizes intranuclear lamin A/C speckles; mAb LA-2B3, which stains lamin A/C at the nuclear periphery; and

LB-P, which is a rabbit polyclonal antibody to lamin B1 (Jagatheesan *et al.*, 1999). A rabbit polyclonal antibody raised to full-length recombinant lamin A (LA-P) was also used in some experiments to stain the nuclear rim. SC-35 mouse mAb (Fu and Maniatis, 1990) was provided by J. Gall (Carnegie Institution of Washington, Baltimore, MD). Rabbit polyclonal antibodies to MyoD, p21, and His tag, and a mouse mAb to myc tag were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibodies to cyclin D3 (cell cycle I sampler kit) and GRIP-1 (TIF2) were from BD Transduction Laboratories (Lexington, KY). A rabbit polyclonal antibody to MEF2C and a pRb antibody kit were from Cell Signaling Technology (Beverly, MA), and a mouse mAb to E1A was from BD PharMingen (San Diego, CA). Myosin heavy chain clone A41025 was provided by H. Blau (Stanford University, Stanford, CA) and myogenin clone F5D (developed by W. E. Wright, University of Texas) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). A rabbit polyclonal antibody to the HA tag was from Sigma Chemical Co. (St. Louis, MO). A mouse mAb to emerin (clone 4G5) was obtained from Novocastra Laboratories (Newcastle upon Tyne, United Kingdom).

### Immunofluorescence Microscopy

Cells were washed with phosphate-buffered saline (PBS) and then routinely fixed by treatment with 3.5% formaldehyde for 10 min followed by 0.5% (vol/vol) Triton X-100 for 6 min at room temperature, or alternatively with methanol at  $-20^{\circ}\text{C}$  for 10 min for staining with LA-P or LA-2B3. Cells were then incubated with 0.5% gelatin or 10% horse serum in PBS for 1 h followed by incubation with first antibody for 1 h and then Alexa 488- or FITC-conjugated secondary antibody for 1 h at room temperature for single labeling experiments. For double-labeling experiments, fixed cells were incubated with the first primary antibody followed by species/subtype specific Cy3 conjugate, and then with the next primary antibody and appropriate Alexa 488-conjugated secondary antibody. For triple labeling studies, fixed cells were stained sequentially with p21 antibody and anti-rabbit Alexa 488 conjugate, followed by antimyogenin antibody and anti-mouse Cy5 conjugate, and then mAb LA-2H10 and Cy3-conjugated anti-mouse IgM. Samples were mounted in Vectashield (Vector Laboratories, Burlingame, CA) containing 1  $\mu\text{g}/\text{ml}$  DAPI. There was no cross-reactivity of the fluorescent secondary antibodies in control experiments in which either primary antibody was omitted. Confocal laser-scanning microscopy was carried out on a Meridian Ultima scan head attached to an Olympus IMT-2 inverted microscope (Argon-ion laser; Lake Success, NY) or with a Zeiss LSM510 META confocal microscope (Oberkochen, Germany). Image analysis was done using DASYP master program V4.19 (Meridian Instruments, Kent, WA) or LSM510 META Software (Carl Zeiss), and images were assembled using Adobe Photoshop 5.0 (San Jose, NM). For quantitative estimations of labeled cells analysis was carried out by inspection of  $n = 100$  transfected or untransfected cells per sample.

### Extraction of Nuclei

Cells were extracted by the relatively mild protocol described by De Conto *et al.* (2000) as this was found to be more suitable for cells after transfection. Transfected cells plated on coverslips were rinsed twice with TM buffer (50 mM Tris-HCl, pH 7.5, 3 mM MgCl<sub>2</sub>) and then incubated for 10 min on ice in TM buffer containing 0.4% Triton X-100, 0.5 mM CuCl<sub>2</sub>, and 0.2 mM phenylmethylsulfonyl fluoride. Cells were rinsed and incubated with DNase I (20 U/ml) and RNase A (20  $\mu\text{g}/\text{ml}$ ) for 20 min at  $37^{\circ}\text{C}$  in TM buffer. The samples were then treated with 2 M NaCl for 5 min on ice, washed with TM buffer, fixed with formaldehyde, and stained as described above.

### Immunoblot Analysis

C2C12 myoblasts, cyclin D3-transfected myoblasts and myotubes were harvested, lysed in Laemmli's sample buffer, boiled, and electrophoresed through SDS-10% polyacrylamide gels. Gels were electroblotted onto nitrocellulose membrane filters and blocked overnight in 5% BLOTTO in Tris-buffered saline (TBS) containing 0.05% Tween-20. Filters were incubated with primary antibody for 2 h, followed by peroxidase-conjugated secondary antibody in TBS containing 0.05% Tween-20 for 1 h. Bound antibody was visualized using an ECL kit (Amersham Pharmacia, Piscataway, NJ). Blots probed with antibodies to pRb were processed according to the manufacturer's instructions.

## RESULTS

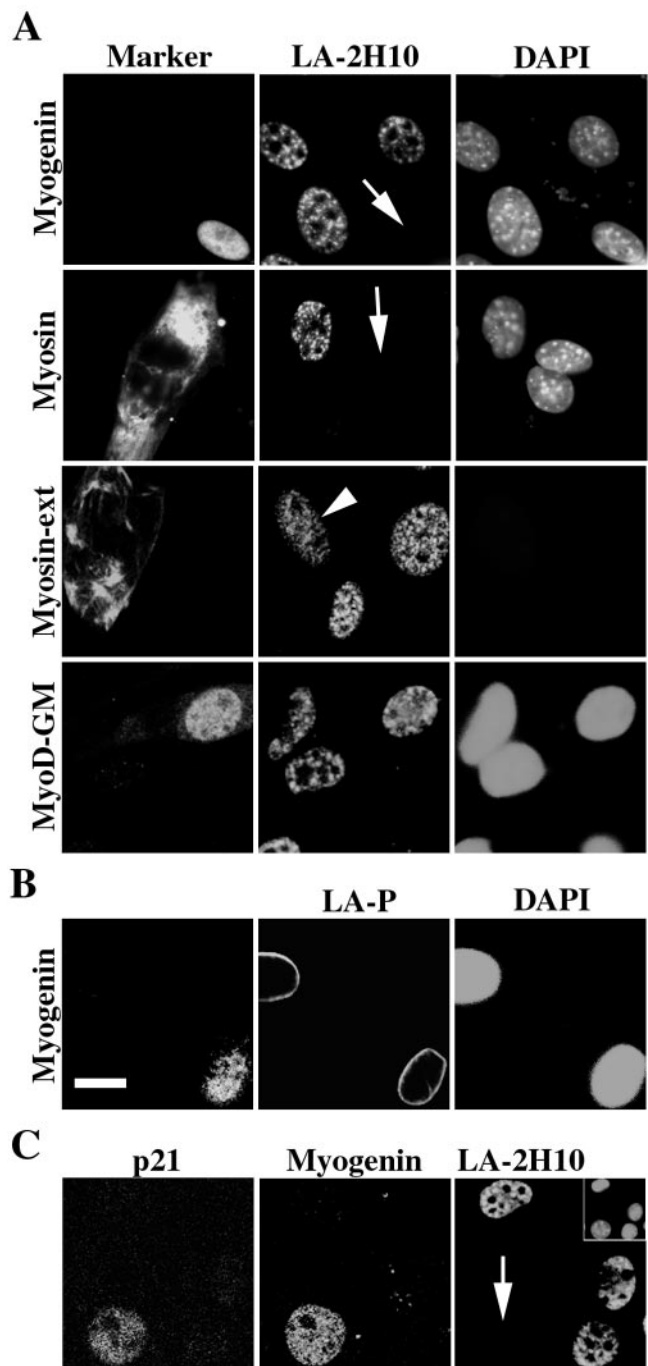
### Reorganization of Lamin A/C Speckles Is Closely Linked to Myogenesis

We initially investigated whether reorganization of lamin A/C speckles was directly linked to the myogenic program by checking if this occurred during MyoD-induced transdifferentiation of fibroblasts. When MyoD was expressed in NIH3T3 cells by transient transfection and cells were then shifted to DM for 48 h, the transfected cells expressed myo-

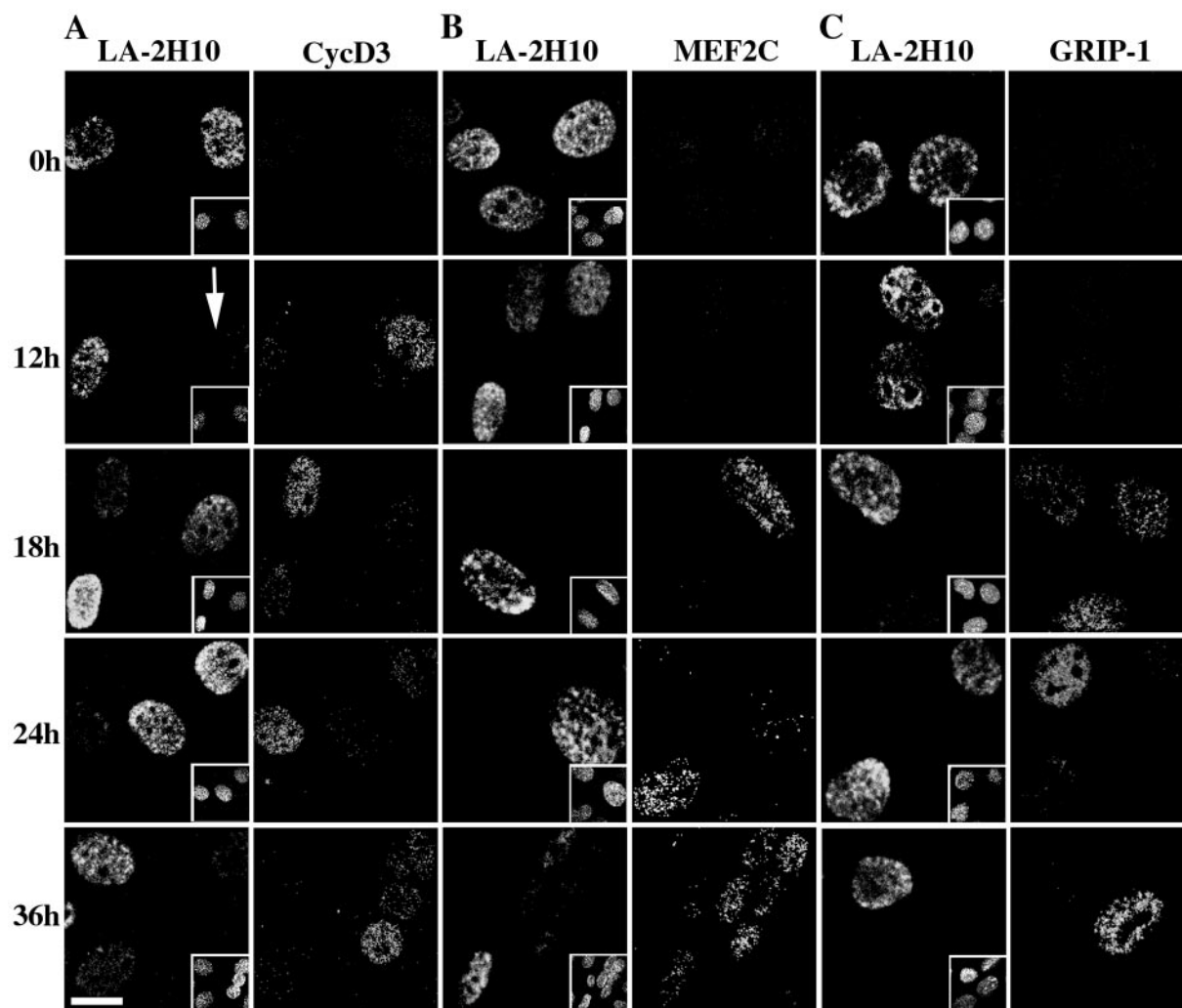
genin and myosin, consistent with earlier reports of the phenomenon (Davis *et al.*, 1987). Lamin A/C speckles stained with mAb LA-2H10 disappeared in the cells positive for myogenin or myosin, but were retained in the surrounding untransfected fibroblasts, as shown in Figure 1A. After extraction of cells with detergent and nucleases, staining of transfected cells with LA-2H10 was recovered. MyoD-transfected cells maintained in GM did not show reorganization of speckles. These experiments confirmed that lamin speckle reorganization was dependent on MyoD induction of the myogenic program. The typical nuclear rim pattern of the A-type lamins was preserved in all cells, as expected (Figure 1B). Similar results were obtained in C3H10T1/2 cells (unpublished data). The expression of the cell cycle inhibitor p21 was induced in transfected cells and, to a lesser extent, also in untransfected fibroblasts because most cells had become quiescent in the low serum conditions of DM. However, lamin speckles were reorganized only in transdifferentiated cells as observed in samples triple-labeled with LA-2H10 and antibodies to p21 and myogenin (see Figure 1C) and not in quiescent fibroblasts. The persistence of lamin speckles in quiescent NIH3T3 cells is consistent with our earlier observations with quiescent C3H10T1/2 cells (Muralikrishna *et al.*, 2001).

#### Lamin Speckle Reorganization Correlates with Onset of Cyclin D3 Expression

Myogenic factors such as MyoD regulate exit from the cell cycle as well as tissue-specific gene expression. Myogenin, MEF2C, and GRIP-1 are essential for muscle-specific gene expression and are expressed early during differentiation (Andrés and Walsh, 1996; Chen *et al.*, 2000). During cell cycle withdrawal, the expression of cyclins D1, A, and E is down-regulated, whereas cyclin D3 is induced several-fold (Kieiss *et al.*, 1995; Rao and Kohtz, 1995; Skapek *et al.*, 1995). MyoD has been shown to directly activate cyclin D3 expression at the onset of muscle differentiation (Cenciarelli *et al.*, 1999). We have earlier shown that the reorganization of lamin A/C speckles occurs at an early stage of the differentiation pathway, starting ~6–12 h after serum deprivation when myogenin is expressed. At 6–12 h, masking of lamin speckles does not strictly correlate with myogenin expression because speckles are still evident in ~50% of myogenin-expressing cells, but this number decreases rapidly by 12–18 h, and postmitotic cells that express p21 do not display lamin speckles (Muralikrishna *et al.*, 2001). To investigate the time course of lamin speckle reorganization and expression of other early markers of differentiation such as cyclin D3, MEF2C, and GRIP-1, C2C12 myoblasts were transferred to DM and examined at 12–36 h by staining with LA-2H10 and antibodies to these markers. As shown in Figure 2, a uniform nuclear staining of cyclin D3 could be detected in cells 12 h after transfer to DM and the number of positive cells increased as differentiation proceeded, concomitantly with a decrease in cells displaying lamin speckles. Importantly, lamin speckles were not observed in cyclin D3-positive cells. A few mononucleated cells were observed to be negative for both lamin speckles and muscle markers at later time points and were likely to be quiescent “reserve” cells as reported earlier (Yoshida *et al.*, 1998). MEF2C and GRIP-1 positive cells were detected only after 18 h in DM, and these cells also did not display lamin speckles. Thus the earliest marker analyzed whose expression coincides with reorganization of lamin A/C speckles is cyclin D3.



**Figure 1.** Lamin speckles are reorganized in transdifferentiated fibroblasts. (A) NIH3T3 cells transfected with MyoD were transferred to DM for 48 h and then stained with LA-2H10 and antibodies to the muscle markers myogenin or myosin. One sample was extracted with detergent and nucleases before staining (Myosin-ext), and another sample was maintained in GM before staining with antibody to MyoD (MyoD-GM). (B) MyoD-transfected cells (in DM) were stained with a polyclonal antibody to lamins A/C (LA-P) and antibody to myogenin. (C) MyoD-transfected cells (in DM) were stained with LA-2H10 and antibodies to p21 and myogenin. Arrows indicate transdifferentiated cells not stained by LA-2H10 and an arrowhead points to an extracted cell showing recovery of LA-2H10 staining. Bar, 10  $\mu$ m.



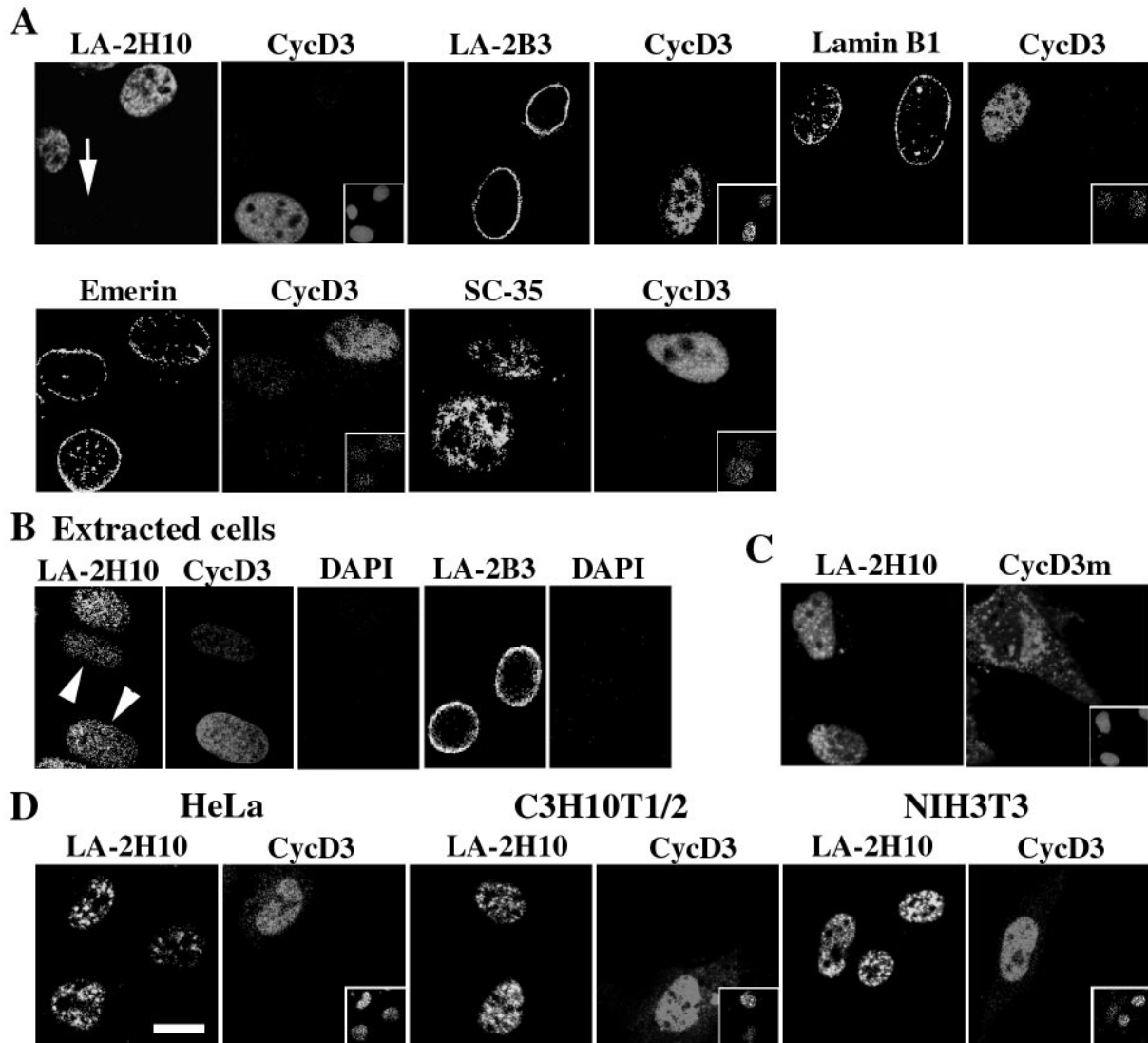
**Figure 2.** Time course of expression of muscle markers and disappearance of lamin speckles. After transfer of C2C12 myoblasts to DM, samples were stained with LA-2H10 and antibodies to endogenous (A) cyclin D3 (CycD3), (B) MEF2C, or (C) GRIP-1 > 0–36 h. Arrow indicates a cyclin D3-positive cell at 12 h, which does not display lamin A/C speckles. DAPI staining of nuclei is shown in insets. Bar, 10  $\mu$ m.

#### *Ectopic Cyclin D3 Induces Reorganization of Lamin A/C Speckles in Myoblasts*

We explored the possibility that ectopic expression of cyclin D3 might reorganize lamin speckles in myoblasts. When HA-tagged cyclin D3 was expressed in proliferating C2C12 cells in GM, it was localized in the nucleus in 80% of cells and was cytoplasmic or pancellular in the remaining cells. Significantly, lamin speckles disappeared in >90% of the transfected cells in which cyclin D3 was localized in the nucleus (see Figure 3A), but not in myoblasts in which cyclin D3 was cytoplasmic. Staining of lamins or emerin at the nuclear rim was not perturbed by expression of cyclin D3. The predominantly speckled staining of the splicing factor SC-35 was also preserved. This is consistent with our earlier observation that SC-35 speckles no longer colocalize with lamin speckles upon lamin reorganization in differentiated muscle cells (Muralikrishna *et al.*, 2001). To investigate whether the disappearance of lamin speckles in cyclin D3-transfected cells was due to their antigen masking as observed previously in differentiated myocytes (Muralikrishna *et al.*, 2001), cells were extracted with detergent and nucle-

ases and then stained with mAb LA-2H10. Staining was recovered in transfected cells after extraction (Figure 3B). Cyclin D3 was also retained in the insoluble matrix of the extracted nuclei. The nuclear rim pattern of lamin staining was not altered by the extraction procedure. The effects of cyclin D3 expression on lamin organization were examined in different cell types. In HeLa, C3H10T $\frac{1}{2}$ , and NIH3T3 cells, ectopically expressed cyclin D3 was also localized in the nucleus in the majority of cells. Interestingly, cyclin D3 expression did not affect lamin speckles in these cell types (Figure 3D). The exogenous expression of p21 in C2C12 myoblasts in GM, which has been reported to stimulate muscle-specific gene expression (Skapek *et al.*, 1995), did not reorganize lamin speckles (unpublished data). Overexpression of cyclin A, E, or D1, which are already present in proliferating cells, also did not alter lamin speckles (unpublished data).

In an experiment designed to examine the requirement for cdk4, which associates with cyclin D3 in differentiated myocytes (Tedesco *et al.*, 1997; Cenciarelli *et al.*, 1999), C2C12 myoblasts were transfected with a mutant cyclin D3 (K112E)



**Figure 3.** Expression of ectopic cyclin D3 induces reorganization of lamin speckles. (A) C2C12 myoblasts were transfected with an HA-tagged cyclin D3 expression vector (in GM) and stained with HA antibody and mAb LA-2H10 or other indicated antibodies. Arrow indicates a transfected cell that does not display lamin A/C speckles. (B) A sample of transfected C2C12 cells was extracted with detergent and nucleases before staining with mAb LA-2H10 and HA antibody. Extracted cells were also stained with mAb LA-2B3. Arrowheads show transfected, extracted cells stained with LA-2H10. (C) C2C12 cells transfected with the cyclin D3 K112E mutant (CycD3m) were stained with LA-2H10 and HA antibody. (D) HeLa, C3H10T $\frac{1}{2}$ , and NIH3T3 cells were transfected with the cyclin D3 construct and stained with LA-2H10 and HA antibody. DAPI staining of nuclei is shown in insets in A, C, and D. Bar, 10  $\mu$ m.

construct which cannot bind to and activate cdk4 (Lazaro *et al.*, 2002). Mutant cyclin D3 was located in the cytoplasm of transfected cells and was unable to induce reorganization of lamin speckles (see Figure 3C), suggesting that binding to or activating cdk4 is required for nuclear targeting (or retention) of cyclin D3 and its subsequent effects on lamin organization. It may be noted that the K112E mutation in the kinase-binding site of cyclin D3 might also affect binding to other kinases such as cdk2, which has been shown to bind to cyclin D3 (Ewen *et al.*, 1993; Tedesco *et al.*, 1997). Coexpression of cdk4 with wild-type cyclin D3 in C2C12 myoblasts did not result in any additional changes (unpublished data), probably because endogenous cdk4 protein is constitutively expressed in both proliferating and differentiated myocytes (Skapek *et al.*, 1995); cdk4 interacts with cyclin D1 in proliferating myoblasts, but upon differentiation it forms com-

plexes with cyclin D3 and p21 (Tedesco *et al.*, 1997; Cenciarelli *et al.*, 1999). Taken together the above experiments suggest that lamin rearrangements are induced by cyclin D3 when it is nuclear localized in C2C12 cells but not in other cell types.

We then confirmed that ectopic cyclin D3-expressing cells were competent to enter the normal differentiation program. In transfected cells maintained in GM, MyoD levels were not significantly altered and none of the cells expressed myogenin, or up-regulated GRIP-1 or MEF2C as checked by immunofluorescence assays. RNA polymerase II transcription was also not altered in cyclin D3-expressing cells (unpublished data). On transfer to DM, cyclin D3-transfected cells expressed myogenin and fused to form myotubes as observed with untransfected cells. A higher percentage of cells were positive for myogenin in cyclin D3-expressing cells at

**Table 1.** Analysis of myogenin expression

	0 h	4 h	8 h	12 h	24 h
Untransfected cells	1.61 ± 0.04	3.21 ± 0.86	5.25 ± 0.80	8.81 ± 1.53	14.08 ± 1.25
Cyclin D3-transfected cells	1.58 ± 0.04	7.27 ± 0.37	11.33 ± 0.84	21.18 ± 2.05	39.40 ± 3.86

Percentage of cells positive for myogenin after transfer to DM. Data represent the mean ± SD of three experiments

each time point (shown in Table 1), suggesting that these cells had entered the differentiation program ahead of the untransfected cells.

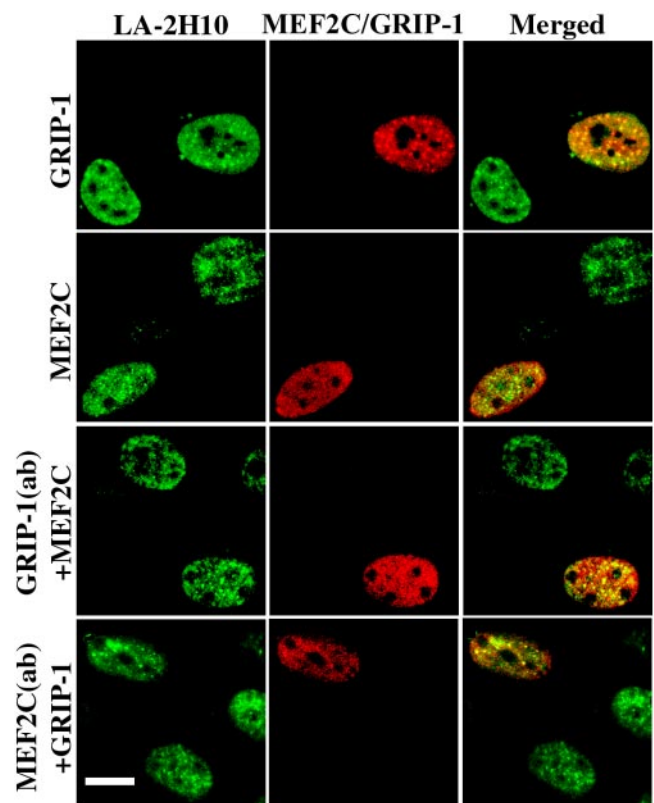
#### Ectopic GRIP-1 and MEF2C Do Not Alter Lamin Speckles

GRIP-1 and MEF2C have been observed to distribute to nuclear foci in differentiated myotube nuclei or upon over-expression in C3H10T $\frac{1}{2}$  cells (Chen *et al.*, 2001; Lazaro *et al.*, 2002), but the functional significance of this localization is not clear. As the GRIP-1 and MEF2C foci were reminiscent of lamin speckles, we explored the possibility that ectopically expressed GRIP-1 or MEF2C might localize to and/or disrupt speckles in C2C12 cells. Cells were transfected with expression vectors for GRIP-1 and/or MEF2C and doubly stained with the corresponding antibodies and mAb LA-2H10. In cells expressing GRIP-1 and MEF2C together or alone, these factors were localized diffusely as well as in punctate structures or nuclear speckles that mostly colocalized with lamin speckles. Importantly, lamin speckles were not altered in the majority of cells (~70%) ectopically expressing MEF2C and GRIP-1 (see Figure 4), though some dispersal of speckles was observed in the remaining cells. This suggests that the expression of these factors is not sufficient to reorganize speckles, although lamin speckles are not evident in differentiating myocytes expressing MEF2C or GRIP-1 after 18 h in DM (see Figure 2). These results are consistent with the reorganization of speckles being mediated by a factor(s) that is expressed before MEF2C or GRIP-1 during differentiation, that is, by cyclin D3, as suggested by the experiments in the previous section. Expression of MEF2C and GRIP-1 in C3H10T $\frac{1}{2}$  or HeLa cells also did not affect lamin speckles and a constitutively active calcium, calmodulin-dependent protein kinase I (CaMKI), which has been shown to activate MEF2C by dissociating it from histone deacetylases (McKinsey *et al.*, 2000), did not affect lamin speckles either (unpublished data).

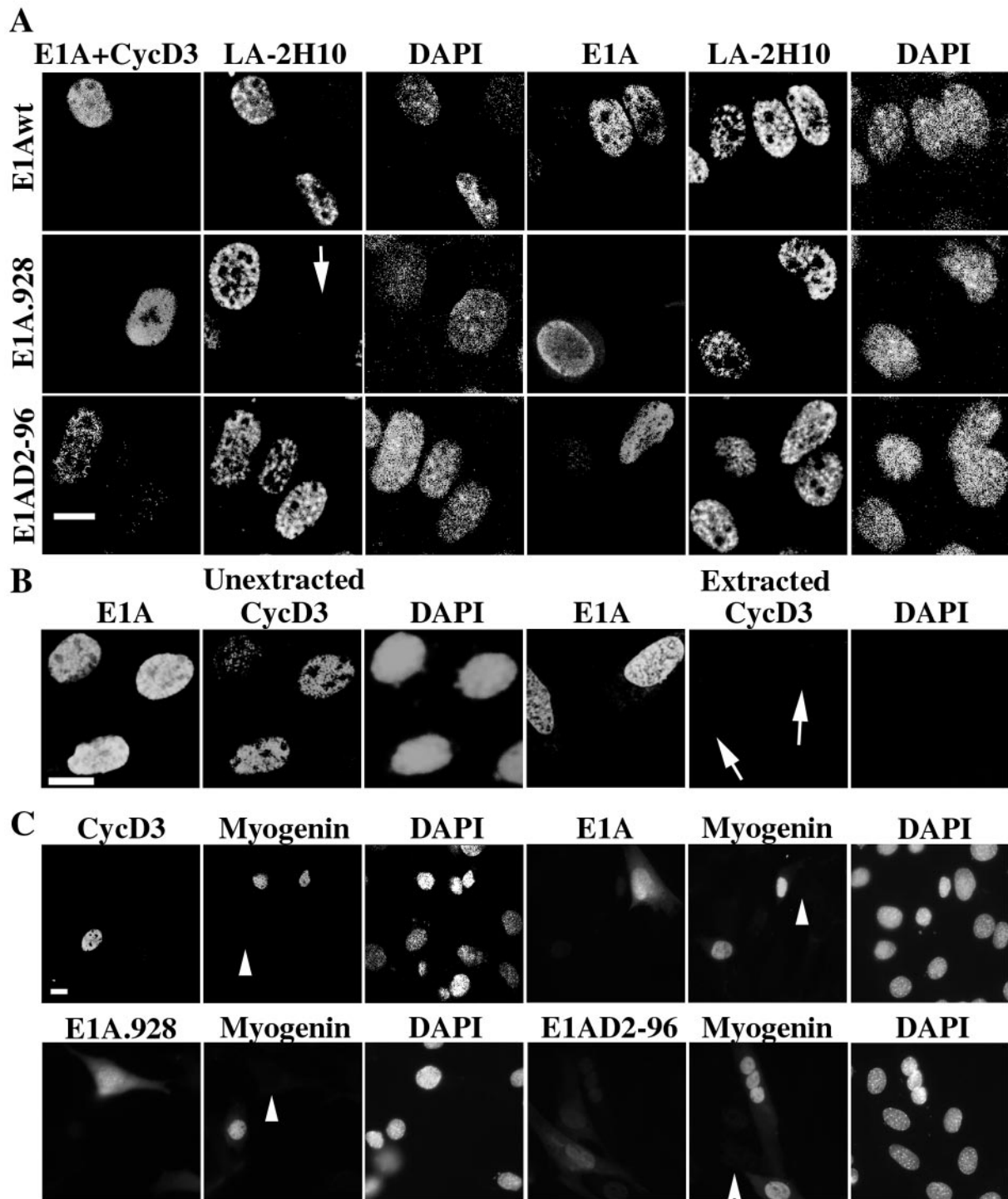
#### Reorganization of Lamin Speckles by Cyclin D3 Requires pRb

Because muscle differentiation is associated with activation of pRb, and cyclin D3 complexes with pRb during differentiation (Dowdy *et al.*, 1993; Ewen *et al.*, 1993), we explored the possibility that reorganization of lamin speckles by cyclin D3 might require pRb. The binding of D-type cyclins to pRb and pRb-family members can be disrupted by viral oncoproteins such as E1A because their pRb-binding motifs are similar. To examine a possible association between the presence of pRb and lamin reorganization, the effects of coexpressing E1A or its mutants (Mal *et al.*, 2000) and cyclin D3 in myoblasts in GM were determined and the results are shown in Figure 5A. It was observed that lamin speckles were intact in >90% of cells expressing wild-type E1A and cyclin D3 in nuclei. Significantly, in cells expressing cyclin D3 and a mutant of E1A, which was unable to bind to pRb (E1A.928), lamin speckles were masked in 70% of cells. However, in cells expressing cyclin D3 and an E1A mutant

(E1AD2-36), which could not bind to p21 but was able to sequester pRb, lamin speckles were masked only in 20% of cells. Hence the reorganization of speckles by cyclin D3 depends on the availability of pRb, but prior expression of p21 is not essential. In myoblasts transfected with only wild-type E1A or its mutants in GM, there were no significant changes in lamin speckle organization. When cells were transferred to DM after transfection and observed after 36 h, E1A inhibited myoblast differentiation as documented earlier (Webster *et al.*, 1988) and cells expressing E1A retained lamin speckles and underwent DNA synthesis as expected (unpublished data). Because we had observed that cyclin D3 was bound to the insoluble matrix in cyclin D3-transfected myoblasts (see Figure 3B), we checked the solubility of cyclin D3 in cells transfected with both cyclin D3 and wild-



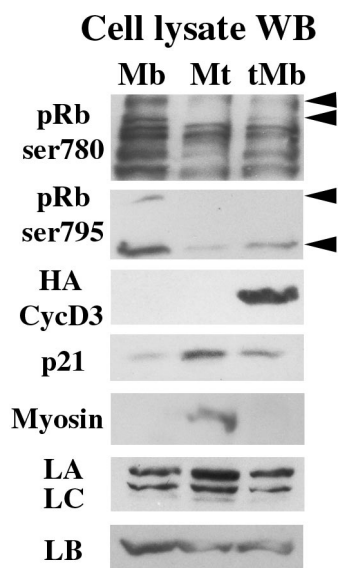
**Figure 4.** Ectopic GRIP-1 and MEF2C localize in speckles. C2C12 myoblasts were transfected with GRIP-1 and/or MEF2C expression vectors (in GM) and stained with antibodies to GRIP-1 or MEF2C, and LA-2H10. For cells transfected with both GRIP-1 and MEF2C, one sample was stained with GRIP-1 antibody and another with MEF2C antibody as indicated. Colocalized speckles are apparent as yellow spots in the merged panels. Similar data were obtained with antibodies to the epitope tags of GRIP-1 or MEF2C (unpublished data). Bar, 10  $\mu$ m.



**Figure 5.** Reorganization of lamin speckles requires pRb. (A) C2C12 myoblasts were transfected with cyclin D3 and/or E1A constructs (in GM) and stained with antibody to E1A and LA-2H10. Arrow indicates a cell transfected with cyclin D3 and E1A.928 (unable to bind to pRb), which does not show LA-2H10 staining. Similar results were obtained by using an antibody to the HA tag of cyclin D3 instead of E1A antibody. (B) Cells transfected with cyclin D3 and E1A (wild-type) were extracted with detergent and nucleases and stained (before and after extraction) with antibodies to E1A and HA. Arrows indicate the absence of cyclin D3 in extracted, transfected cells. (C) Myoblasts in GM were transfected with cyclin D3 or E1A constructs and counterstained with myogenin antibody. Arrowheads indicate myogenin-negative transfected cells. Areas enriched in myogenin-positive untransfected cells are displayed so as to provide positive controls for staining; only ~5% of total untransfected myoblasts in GM were positive for myogenin. Bar, 10  $\mu$ m.

type E1A. On detergent extraction and nuclease treatment, cyclin D3 could not be detected in E1A-positive cells (Figure 5B). In a control experiment it was confirmed that expression

of cyclin D3 or E1A constructs did not induce expression of the differentiation marker myogenin (shown in Figure 5C). Taken together, our results suggest that cyclin D3 is seques-



**Figure 6.** Cyclin D3 induces hypophosphorylation of pRb. Whole cell lysates of C2C12 myoblasts (Mb), myotubes (Mt), and cyclin D3-transfected myoblasts (tMb) in GM were analyzed by Western blotting with antibodies to pRbser780 and 795, HA tag of cyclin D3 (HACycD3), p21, myosin, lamin B1 (LB), and mAb LA-2H10 (LA, LC). The phosphorylated species of pRb, which are dephosphorylated in myotubes and transfected myoblasts, are indicated by arrowheads.

tered into the insoluble matrix upon reorganization of internal lamins in a process that requires pRb.

As it has been observed that myotube nuclei can partially reenter G1 and S phases of the cell cycle in response to growth factors or expression of E1A (Tiainen *et al.*, 1996), we checked mAb LA-2H10 reactivity in myotube cultures incubated with GM or transfected with E1A. Lamin speckles were not restored by these treatments (unpublished data), suggesting that the reorganization of internal lamins in myotube nuclei is an irreversible event.

#### *pRb Is Hypophosphorylated in Cyclin D3-transfected Myoblasts*

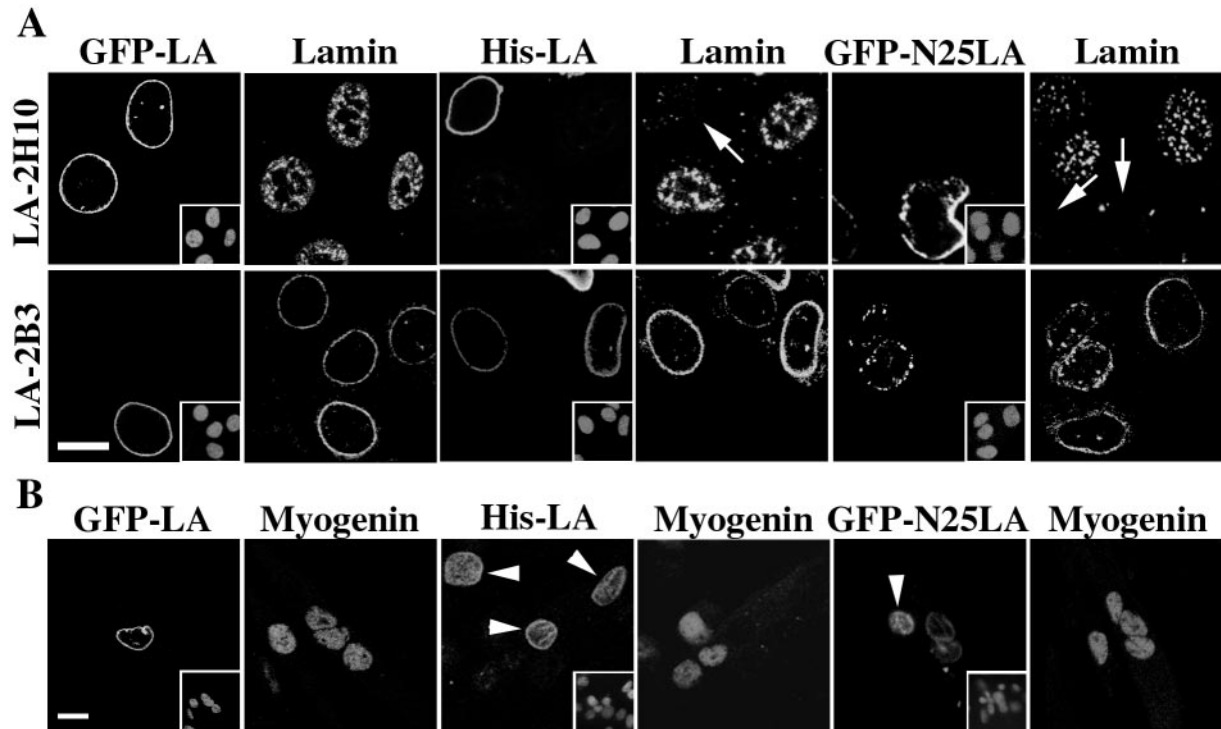
It has been previously shown that pRb becomes hypophosphorylated and resistant to detergent extraction as differentiating cells withdraw from the cell cycle (Thornburn *et al.*, 1993). To determine whether changes in pRb phosphorylation can be induced by ectopic expression of cyclin D3 in myoblasts, whole cell lysates were analyzed by Western blotting with antibodies specific for the different phosphorylated forms of pRb. The data indicated a significant reduction in ser795 phosphorylation in cyclin D3-transfected myoblasts as well as myotubes compared with untransfected myoblasts (Figure 6). With the antibody to ser780 which recognizes multiple forms of phosphorylated pRb (the slower mobility forms being more phosphorylated), two slower mobility forms were absent in myotubes and cyclin D3-transfected myoblasts but present in untransfected myoblasts. Hence cyclin D3 expression in myoblasts leads to dephosphorylation of pRb at specific sites that appear to correspond to sites that are dephosphorylated in myotubes. Furthermore, there was an increase in levels of p21 though the differentiation-specific late marker myosin was not expressed in the transfected myoblasts. Levels of lamins were not significantly altered.

#### *Disruption of Lamin Speckles Inhibits Expression of Myogenin*

We sought to determine whether perturbation of the lamina, in particular lamin speckles, had an effect on myoblast differentiation. For this purpose, we examined the effects of overexpression of various lamin constructs on expression of the muscle regulatory factor myogenin and also assessed effects on the lamina. As shown in Figure 7A, a GFP fusion with full-length lamin A (GFP-lamin A) was correctly incorporated into the nuclear rim in C2C12 myoblasts, consistent with earlier studies using a similar construct (Izumi *et al.*, 2000; Moir *et al.*, 2000). GFP-lamin A did not disrupt the endogenous peripheral lamina or lamin speckles, but was also not incorporated into speckles and hence could not be used for tracking speckles in living cells. An His epitope-tagged full-length lamin A (His-lamin A) was incorporated into the nuclear rim and did not perturb the peripheral lamina; however, expression of His-lamin A resulted in loss of speckles in the majority of cells due to their disruption; we have checked in an earlier study with HeLa and NIH 3T3 cells that this loss of speckles is not due to masking (Kumaran *et al.*, 2002). The ability of lamin constructs that lack the amino or carboxy terminal segments to act as dominant negative proteins and disrupt the peripheral lamina and form nucleoplasmic aggregates has been well documented (Spann *et al.*, 1997; Izumi *et al.*, 2000; Moir *et al.*, 2000). We observed that the expression of a GFP fusion with lamin A bearing an amino terminal deletion of 25 amino acids (GFP-N25lamin A) led to loss of lamin speckles, though in cells expressing lower amounts of the fusion protein speckles were mostly intact. GFP-N25lamin A was partially incorporated into the peripheral lamina and also formed aggregates. As shown earlier in other cell types (Kumaran *et al.*, 2002; Spann *et al.*, 2002), expression of His-lamin A or GFP-N25lamin A in myoblasts down-regulated RNA polymerase II transcription (unpublished data). Myoblasts expressing the above constructs were transferred to DM and the percentage of surviving cells and their ability to express myogenin was evaluated after 36 h in DM. As shown in Table 2, GFP-lamin A-expressing cells showed the maximum survival, followed by His-lamin A and GFP-N25lamin A. With GFP-N25lamin A, cell death was three times higher than with GFP-lamin A.

The majority of surviving GFP-lamin A-transfected cells expressed myogenin and fused into myotubes. Interestingly, most of the His-lamin A-transfected cells were unable to express myogenin and remained in a mononucleated state though the peripheral lamina was clearly intact. With GFP-N25lamin A, only those transfected cells that expressed the fusion protein at low levels in a nearly normal peripheral lamina pattern expressed myogenin and fused to form myotubes, whereas cells with aggregates did not express myogenin. These data are illustrated in Figure 7B. With both His-lamin A and GFP-N25lamin A, a small proportion of transfected cells not expressing myogenin fused with normal cells to form myotubes, suggesting that expression of these constructs might not have a significant effect on membrane fusion events required for myotube formation though it prevents expression of early differentiation markers, thereby leading to incomplete or aberrant differentiation. A GFP fusion construct having a deletion of the carboxy terminal 56 residues of lamin A showed strong dominant negative effects as it formed large aggregates and disrupted the peripheral lamina and speckles; most transfected cells died upon transfer to DM, and the remaining cells did not express myogenin (unpublished data). Our results indicate that per-





**Figure 7.** Loss of lamin speckles inhibits myogenin expression. (A) C2C12 myoblasts in GM were transfected with GFP-lamin A (GFP-LA), His-lamin A (His-LA), or GFP-N25lamin A (GFP-N25LA) and stained with lamin antibodies LA-2H10 or LA-2B3. His-lamin A-positive cells were stained with an antibody to the His epitope. Arrows indicate transfected cells that do not display lamin speckles. (B) Transfected myoblasts were transferred to DM and stained for myogenin after 36 h. Arrowheads point to transfected cells that do not express myogenin. Bar, 10  $\mu$ m.

turbation of the lamina and, in particular, lamin speckles inhibits expression of the essential muscle regulatory factor myogenin.

## DISCUSSION

During myoblast differentiation, myogenic regulatory factors control the expression of muscle-specific markers as well as cell cycle arrest. The key myogenic regulator MyoD promotes cell cycle arrest by regulating cyclin D3, pRb, and p21 levels (Hallevy *et al.*, 1995; Cenciarelli *et al.*, 1999). Muscle differentiation is accompanied by changes in nuclear architecture as intranuclear lamin A/C speckles are reorganized and antigenically masked in postmitotic cells (Muralikrishna *et al.*, 2001). In this study we show that lamin reorganization is induced specifi-

cally in muscle cells by expression of cyclin D3 and requires the presence of pRb. We provide additional evidence that this reorganization is controlled by the myogenic program because it can be brought about in fibroblasts triggered to transdifferentiate to myoblasts by MyoD. Furthermore, disruption of lamin structures by overexpression of lamin constructs prevents expression of myogenin. A summary of our findings is presented in a flow chart in Figure 8.

### *Cyclin D3 Mediates Lamin Reorganization in Muscle Cells*

Cyclin D3 behaves as an atypical cyclin in muscle cells as it is activated several-fold when myoblasts are transferred to serum-depleted medium to induce differentiation (Kieiss *et al.*, 1995; Rao and Kohtz, 1995) and is expressed at high levels during the early stages of mouse muscle development

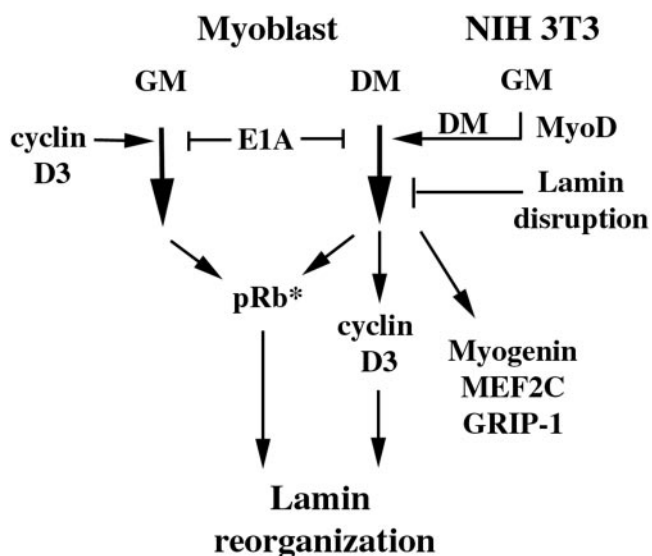
**Table 2.** Effects of ectopic lamins on myogenin expression

Construct	% Transfected cells in DM <sup>a</sup>	% Myogenin-positive cells		% Myogenin-negative cells	
		Mononucleated	Myotube	Mononucleated	Myotube
GFP-Lamin A	49.68 $\pm$ 3.78	10.96 $\pm$ 1.58	33.45 $\pm$ 1.21	5.28 $\pm$ 2.58	0.00
His-Lamin A	27.48 $\pm$ 0.84	0.00	0.81 $\pm$ 0.58	22.29 $\pm$ 0.59	4.37 $\pm$ 0.45
GFP-N25LA	16.47 $\pm$ 2.83	4.10 $\pm$ 1.46 <sup>b</sup>	10.21 $\pm$ 1.14 <sup>b</sup>	0.27 $\pm$ 0.48	1.88 $\pm$ 0.32
Untransfected	na <sup>c</sup>	13.68 $\pm$ 1.51	41.52 $\pm$ 4.57	44.80 $\pm$ 6.08	0.00

<sup>a</sup>Normalized to transfection efficiency in GM. Data represent the mean  $\pm$  SD of three experiments.

<sup>b</sup>Low levels of expression of GFP-N25LA only.

<sup>c</sup>Not applicable.



**Figure 8.** Summary of factors regulating lamin reorganization in muscle cells. In C2C12 myoblasts, cyclin D3, either ectopically expressed in GM or endogenously in DM, and the hypophosphorylated form of pRb (pRb\*) produced under these conditions together mediate lamin reorganization. Expression of MyoD in NIH 3T3 cells in GM followed by transfer to DM to allow transdifferentiation to muscle cells also induces lamin reorganization. Differentiation and lamin reorganization are inhibited by E1A (by sequestration of pRb) and by the overexpression of lamin constructs that disrupt lamin structures. Other early differentiation markers such as myogenin, MEF2C, and GRIP-1 do not play a direct role in lamin reorganization.

in vivo (Bartkova *et al.*, 1998). Cyclin D3 has been reported to sequester pRb, cdk2, cdk4, p21, and PCNA into extraction-resistant, kinase-inactive complexes in differentiated muscle cells (Kiess *et al.*, 1995; Tedesco *et al.*, 1997; Cenciarelli *et al.*, 1999). Further, cyclin D3 levels are elevated in a number of differentiated cell types, and a role for cyclin D3 in the induction and/or maintenance of terminal differentiation has been proposed (Bartkova *et al.*, 1998). Most studies on the biological effects of cyclin D3 in muscle cells have been carried out on the endogenous gene or by overexpression of cyclin D3, as cyclin D3-knock out mice are born normal, probably due to compensation by other D-type cyclins, and show defects primarily in T-cell development later in life (Sicinska *et al.*, 2003). In nonmuscle cell lines ectopic expression of cyclin D3 can modulate the association of exogenously expressed GRIP-1 with MEF2C in speckles (Lazaro *et al.*, 2002). We have observed that ectopic expression of cyclin D3 in myoblasts causes antigen masking and reorganization of lamin A/C speckles in a process that requires pRb. Further, cyclin D3-expressing cells are competent to differentiate. On the basis of our results we propose that lamin reorganization by cyclin D3 is an important regulatory step in muscle differentiation.

#### Requirement of pRb for Lamin Reorganization

pRb plays an essential role in events leading to cell cycle withdrawal during muscle differentiation as well as in expression of late markers of differentiation (Gu *et al.*, 1993; Schneider *et al.*, 1994). Although pRb null myocytes fuse into myotubes, differentiation is incomplete as cell cycle arrest is reversible and expression of muscle markers is affected (Novitsch *et al.*, 1996). During differentiation pRb becomes hypo-

phosphorylated and associates with cyclin D3 in kinase-inactive complexes on insoluble nuclear structures (Cenciarelli *et al.*, 1999). The sequestration of pRb by viral oncoproteins such as E1A has been valuable for understanding pRb function (Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Cenciarelli *et al.*, 1999; Mal *et al.*, 2000). Our experiments on the coexpression of cyclin D3 and E1A or its mutants in myoblasts reveal that the ability of cyclin D3 to reorganize internal lamins depends on the availability of pRb. When pRb is sequestered by E1A, lamin speckles are not reorganized by cyclin D3, and cyclin D3 is not resistant to detergent extraction. But it may be noted that pRb alone cannot initiate these changes because they do not occur in the absence of cyclin D3 expression in myoblast nuclei. Our in vitro analysis has shown that in myotubes and myoblasts transfected with cyclin D3, pRb is hypophosphorylated. It is possible that the triggering of an event such as kinase inactivation or phosphatase activation by cyclin D3 may result in hypophosphorylation and sequestration of pRb.

There is considerable evidence supporting the specific binding of A-type lamins to pRb. Hypophosphorylated pRb has been shown to bind to the peripheral and internal nuclear matrix of cells in a cell cycle-dependent manner in the early G1 phase, and lamin C has been identified as a pRb-binding protein in a screen with a cDNA expression library (Mittnacht and Weinberg, 1991; Mancini *et al.*, 1994). In vitro studies using blot binding assays have demonstrated direct binding of pRb to a specific segment of lamin A (Ozaki *et al.*, 1994). (This binding region is ~100 amino acid residues downstream to the epitope sequence for mAb LA-2H10.) Another component of the lamina, lamina-associated polypeptide 2 $\alpha$  can also bind to pRb and tether it in the nucleus (Markiewicz *et al.*, 2002). The nuclear anchorage of pRb has been proposed to be important for its function, as pRb deletion mutants identified in certain tumors are not anchored in the nucleus (Mittnacht and Weinberg, 1991; Mancini *et al.*, 1994) and are unable to bind to lamins A/C or lamina-associated polypeptide 2 $\alpha$  (Markiewicz *et al.*, 2002). These reports together with our detergent extraction data lend support to our view that the sequestration of cyclin D3 and pRb on a lamin-containing matrix is required for muscle differentiation.

#### Possible Functional Roles of Lamin Reorganization in Muscle Differentiation

The adaptation of the nucleus of the dividing myoblast to the postmitotic myotube is associated with changes in nuclear organization. For instance, the nuclear mitotic apparatus protein, NuMa, which is a component of the internal matrix in interphase nuclei and plays an important role during mitosis (Lydersen and Pettijohn, 1980), is progressively degraded during myotube formation though it is expressed in other differentiated tissues (Merdes and Cleveland, 1998). A possible role for the reorganization of the nuclear substructure in myotube nuclei might be associated with their postmitotic state. The pathways for maintaining quiescence in myotube nuclei are unique, because exogenous E2F1 is unable to induce DNA synthesis in these nuclei though it can promote entry into S phase in other cell types (Tiainen *et al.*, 1996). The sequestration of hypophosphorylated pRb on the insoluble matrix is an essential requirement for cell cycle exit (Cenciarelli *et al.*, 1999), and because this event coincides with the reorganization of lamin speckles, it is possible that the antigenically masked lamin network of the myotube nucleus is necessary for its nondividing status.

It is becoming increasingly evident that a functional lamin network is essential for myoblast differentiation. In a recent

report, ectopic expression of a mutant lamin A bearing the R453W mutation, which is known to cause EDMD in humans, has been shown to inhibit differentiation of C2C12 myoblasts and lead to apoptosis (Favreau *et al.*, 2004). In the present study we have observed that disruption of the lamina, in particular lamin speckles, prevents expression of the essential muscle regulatory factor myogenin. Because lamin disruption also results in down-regulation of transcription, the inhibitory effects on differentiation may be attributed to a block in lamin reorganization as well as a decrease in transcription. A recent report suggests that lamin deficiency is associated with defective nuclear mechanics and impaired gene transcription (Lammerding *et al.*, 2004). We have earlier proposed a dynamic role for lamin speckles in the spatial organization of transcription and splicing (Kumaran *et al.*, 2002). In muscle cells the internal lamins appear to perform additional functions by mediating events leading to terminal differentiation. The exact significance of our observation that SC-35 domains are no longer colocalized with lamin speckles in differentiating myocytes unlike other cell types is not clear, but we speculate that this association might not be required in terminally differentiated cells having a predetermined and irreversible program of gene transcription. Hence the role of the internal lamins might be modulated by the functional requirements of the cell type.

#### **Implications of Muscle-specific Changes in Lamin Organization**

The reorganization of lamin speckles leading to antigen masking is likely to involve at least two events: 1) a physical rearrangement of lamins from a speckled pattern to a diffuse network, and 2) protein binding, because epitope reactivity is regained after treatment with detergent, nucleases, and salt, which remove chromatin and nucleoplasmic proteins. The trigger for lamin rearrangement may be phosphorylation of lamins and/or lamin-binding proteins. Changes in phosphorylation have been observed to rearrange lamin speckles because overexpression of Clk/Sty protein kinase results in dispersal of splicing factor domains and lamin speckles (Sacco-Bubulya and Spector, 2002), and lamin speckles are redistributed to a diffuse lamin network, after treatment of various cell types with the phosphatase inhibitor okadaic acid (Mariappan and Parnaik, unpublished work). However, in both cases splicing factor domains are also dispersed and the effects are not cell-type specific. Because nuclear cyclin D3 and pRb specifically induce lamin reorganization in muscle cells and are associated with the diffuse lamin network, a likely possibility is that binding of a complex of cyclin D3/cdk4/pRb and unknown muscle-specific protein(s) to internal lamin A/C might directly or indirectly mediate lamin phosphorylation and rearrangement to a network that is subsequently masked by chromatin or other nucleoplasmic proteins.

A large family of proteins containing spectrin repeats termed the Syne/ANC-1 proteins have been localized in the nuclear envelope of a variety of cell types, and some of these proteins can bind to lamins as well as actin; proteins such as Syne-1 and Myne-1 are primarily expressed in skeletal muscle cells (Mislow *et al.*, 2002; Zhang *et al.*, 2002). Further, emerin-actin interactions are regulated in differentiating myoblasts (Lattanzi *et al.*, 2003). Lamins and their associated proteins are known to play important roles in DNA replication and transcription; actin and actin-binding proteins have been shown to be components of chromatin-remodeling complexes regulating gene activity and are likely to be involved in various aspects of mRNA metabolism (reviewed by Shumaker *et al.*, 2003). Molecular interactions between

these two nucleoskeletal systems may thus lead to specific programs of gene expression in different cell types.

Our results demonstrate for the first time that changes in internal lamin A/C organization in muscle cells are brought about by a specific signaling molecule and key regulator of muscle differentiation, cyclin D3, with the involvement of pRb. We suggest that if this reorganization is hindered by mutant lamins or absence of lamins *in vivo*, it could lead to aberrant differentiation and muscle-specific disease symptoms. An understanding of the functional role of internal lamins in different cell types should thus assist in gaining insights into the complex diseases that result from different mutations in the *LMNA* gene.

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