Lamin A/C and emerin are critical for skeletal muscle satellite cell differentiation

Richard L. Frock,[1] Brian A. Kudlow,[2] Angela M. Evans,[1] Samantha A. Jameson,[1] Stephen D. Hauschka,[1,3,4] and Brian K. Kennedy[1,3,5]

[1]Department of Biochemistry, [2]Molecular and Cellular Biology Program, University of Washington, Seattle, Washington 98195, USA

Mutations within *LMNA*, encoding A-type nuclear lamins, are associated with multiple tissue-specific diseases, including Emery-Dreifuss (EDMD2/3) and Limb-Girdle muscular dystrophy (LGMD1B). X-linked EDMD results from mutations in emerin, a lamin A-associated protein. The mechanisms through which these mutations cause muscular dystrophy are not understood. Here we show that most, but not all, cultured muscle cells from lamin A/C knockout mice exhibit impaired differentiation kinetics and reduced differentiation potential. Similarly, normal muscle cells that have been RNA interference (RNAi) down-regulated for either A-type lamins or emerin have impaired differentiation potentials. Replicative myoblasts lacking A-type lamins or emerin also have decreased levels of proteins important for muscle differentiation including pRB, MyoD, desmin, and M-cadherin; up-regulated Myf5; but no changes in Pax3, Pax7, MEF2C, MEF2D, c-met, and β-catenin. To determine whether impaired myogenesis is linked to reduced MyoD or desmin levels, these proteins were individually expressed in *Lmna*$^{-/-}$ myoblasts that were then induced to undergo myogenesis. Expression of either MyoD or, more surprisingly, desmin in *Lmna*$^{-/-}$ myoblasts resulted in increased differentiation potential. These studies indicate roles for A-type lamins and emerin in myogenic differentiation and also suggest that these effects are at least in part due to decreased endogenous levels of other critical myoblast proteins. The delayed differentiation kinetics and decreased differentiation potential of lamin A/C-deficient and emerin-deficient myoblasts may in part underlie the dystrophic phenotypes observed in patients with EDMD.

[*Keywords*: MyoD; desmin; pRB; myoblast; myogenesis; Emery-Dreifuss muscular dystrophy]

Received August 15, 2005; revised version accepted December 27, 2005.

Nuclear lamins are the major constituents of the nuclear lamina, a proteinaceous network underlying the inner nuclear membrane (Hutchison and Worman 2004; Mounkes and Stewart 2004; Zastrow et al. 2004; Smith et al. 2005). Expression of A-type lamins, in contrast to the homologous B-type lamins, is largely restricted to differentiated tissues with high expression in skeletal muscle (Rober et al. 1989). Lamins A and C (henceforth referred to as lamin A/C) are the major products of the *LMNA* gene.

Greater than 100 mutations in *LMNA* have been linked to 10 human genetic disorders, broadly termed laminopathies (for review, see Mounkes et al. 2003). The majority of *LMNA* mutations identified thus far are linked to Emery-Dreifuss muscular dystrophy (EDMD2/3) (Bonne et al. 1999). Mice lacking lamin A/C develop a form of muscular dystrophy closely resembling EDMD and die by 8 wk from cardiac and skeletal myopathies (Sullivan et al. 1999). Mutations within the gene encoding the lamin A/C-binding protein, emerin (EMD), cause an X-linked form of Emery-Dreifuss muscular dystrophy (EDMD1) (Bione et al. 1994; Gruenbaum et al. 2005). It is not known what role A-type lamins play in skeletal muscle development, let alone how mutations within *LMNA* cause EDMD. One possibility is that mutations that alter lamin A/C's nuclear integrity or impair its function(s) would then result in degeneration of cells under high mechanical stress, such as skeletal muscle (Broers et al. 2004; Lammerding et al. 2004). A second, nonexclusive possibility is that lamin A/C plays a role in muscle differentiation, and alterations in its activity result in delayed or decreased expression of genes important for differentiation and/or the stable maintenance of the differentiated state. Inefficient muscle differentiation may ultimately result in a dystrophic syndrome,

[3]**Members of the Hauschka and Kennedy groups contributed equally to these studies.**
Corresponding authors.
[4]**E-MAIL haus@u.washington.edu; FAX (206) 685-1792.**
[5]**E-MAIL bkenn@u.washingon.edu; FAX (206) 685-1792.**
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1364906.

when the balance between muscle degeneration and renewal becomes skewed.

In adults, the vast majority of new skeletal muscle comes from myogenic precursor cells called satellite cells that require Pax3/Pax7 for their specification and self-renewal (Oustanina et al. 2004; Relaix et al. 2005). These adult stem cells are able to proliferate and produce myoblasts, which in turn are capable of withdrawing from the cell cycle and terminally differentiating into skeletal muscle (for review, see Charge and Rudnicki 2004). A number of transcription factors and structural proteins have been implicated in this transition (Parker et al. 2003; Paulin and Li 2004); for example, the myogenic regulatory factor MyoD is expressed prior to myocyte differentiation (Buskin and Hauschka 1989; Lassar et al. 1989) and, together with MEF2 transcription factors, is crucial for satellite cell terminal differentiation (Sabourin et al. 1999; Yablonka-Reuveni et al. 1999; McKinsey et al. 2002). The retinoblastoma protein (pRB), is likewise important for the proliferation to differentiation transition during myogenesis (Maione et al. 1994; Zacksenhaus et al. 1996; Huh et al. 2004). Although the mechanism is not fully understood, pRB is thought to potentiate MyoD activity during muscle differentiation (Novitch et al. 1996, 1999; Puri et al. 2001; Guo et al. 2003).

Several structural and cell surface proteins also play important roles in terminal differentiation. Desmin, a muscle-specific intermediate filament protein, is one of the first proteins expressed upon satellite cell activation (Lazarides and Hubbard 1976; Kaufman et al. 1991). Its exact role in myogenesis remains unclear; but differentiation is slightly delayed during regeneration in desmin knockout mice (Li et al. 1994; Weitzer et al. 1995; Smythe et al. 2001). M-cadherin, a cell surface adhesion protein, is a marker for satellite cells in vivo, and its experimental perturbation also delays the onset of differentiation (Zeschnigk et al. 1995; Kaufmann et al. 1999).

Do A-type lamins play roles in muscle differentiation? A recent study reported that overexpression of a lamin A EDMD mutation, R453W, inhibits the in vitro differentiation of C2C12 myoblasts (Favreau et al. 2004). Subsequent studies showed that overexpression of a different EDMD mutation, W520S, also inhibited C2C12 myoblast differentiation, and provided evidence that nucleoskeleton remodeling is necessary for skeletal muscle differentiation (Markiewicz et al. 2005). Finally, Arimura et al. (2005) constructed an EDMD mouse carrying H222P mutations in both lamin A alleles and found that adult mice developed muscular dystrophy and exhibited elevated levels of Smads 2 and 4 in cardiac and skeletal muscle nuclei. Here, we focus on an EDMD mouse model in which the lamin A gene has been knocked out (Sullivan et al. 1999), and report that *Lmna*$^{-/-}$ and even *Lmna*$^{+/-}$ myoblasts display a dramatically compromised differentiation potential. *Lmna*$^{+/+}$ cells stably expressing a small interfering RNA (siRNA) targeted to *Lmna* are similarly compromised. Interestingly, myoblasts with siRNA-reduced emerin display a similar differentiation phenotype. Furthermore, myoblasts with reduced lamin A/C or emerin also contain reduced levels of at least four proteins important for differentiation and/or the maintenance of the differentiated state: MyoD, desmin, pRB, and M-cadherin. Exogenous expression of MyoD in *Lmna*$^{-/-}$ myoblasts restores their differentiation kinetics, but more surprisingly, forced expression of desmin is sufficient to restore much of their differentiation potential, suggesting that reduced MyoD and desmin transcription may be primary factors underlying the poor differentiation capacity of cells deficient in A-type lamin function.

Results

Generation of Lmna$^{-/-}$ *myoblasts*

To determine the myogenic potential of cells with reduced lamin A/C function, we generated permanent myoblast cell lines from *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ mouse hind leg muscles (see Materials and Methods). To avoid accidental selection of cells with unique attributes during the generation of permanent myoblast lines, primary cultures were grown at densities that permitted thousands of individual muscle colony-forming cells to proliferate into colonies, while most fibroblasts grew poorly. Previous studies indicate that when most mouse primary satellite-cell-derived muscle colonies are subcloned and individually expanded, their descendents undergo proliferative senescence after 20–40 cell cycles and small numbers of spontaneously transformed myoblasts emerge (Hauschka et al. 1979; Neville et al. 1997). Even after decades of expansion, the descendents exhibit biochemical characteristics that are very close to those of primary satellite cells (Cornelison 1998). Importantly, the cells in each of the permanent cell lines generated are Pax3- and Pax7-positive (see below); thus potentially immortalized fibroblasts did not contribute to the myogenic *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ cell lines. Western analysis confirmed that lamin A and C bands were absent in *Lmna*$^{-/-}$ muscle cells and were reduced ~50% in *Lmna*$^{+/-}$ cells (Fig. 1A).

Lmna$^{-/-}$ *and* Lmna$^{+/-}$ *muscle cells have delayed differentiation kinetics*

The onset of terminal differentiation in satellite cell-derived skeletal muscle cultures is regulated by growth factors. In the presence of bFGF and horse serum, mouse myoblasts grow exponentially. Upon bFGF removal, myoblasts continue cycling for ~6 h as residual mitogenic signals attenuate; cells then stop entering S phase and undergo an irreversible commitment to terminal differentiation. Typically, >90% of the population commits to terminal differentiation as mitogen-deprived cells complete mitosis and enter the G_1/G_0 cell cycle compartment. Committed cells activate expression of muscle-specific genes such as myosin heavy chain (MyHC), repress growth factor receptor expression, and are refractory to subsequent mitogenic stimulation (Clegg et al. 1987; Olwin and Hauschka 1988; Templeton and Hauschka 1992; Angello and Hauschka 1996). The

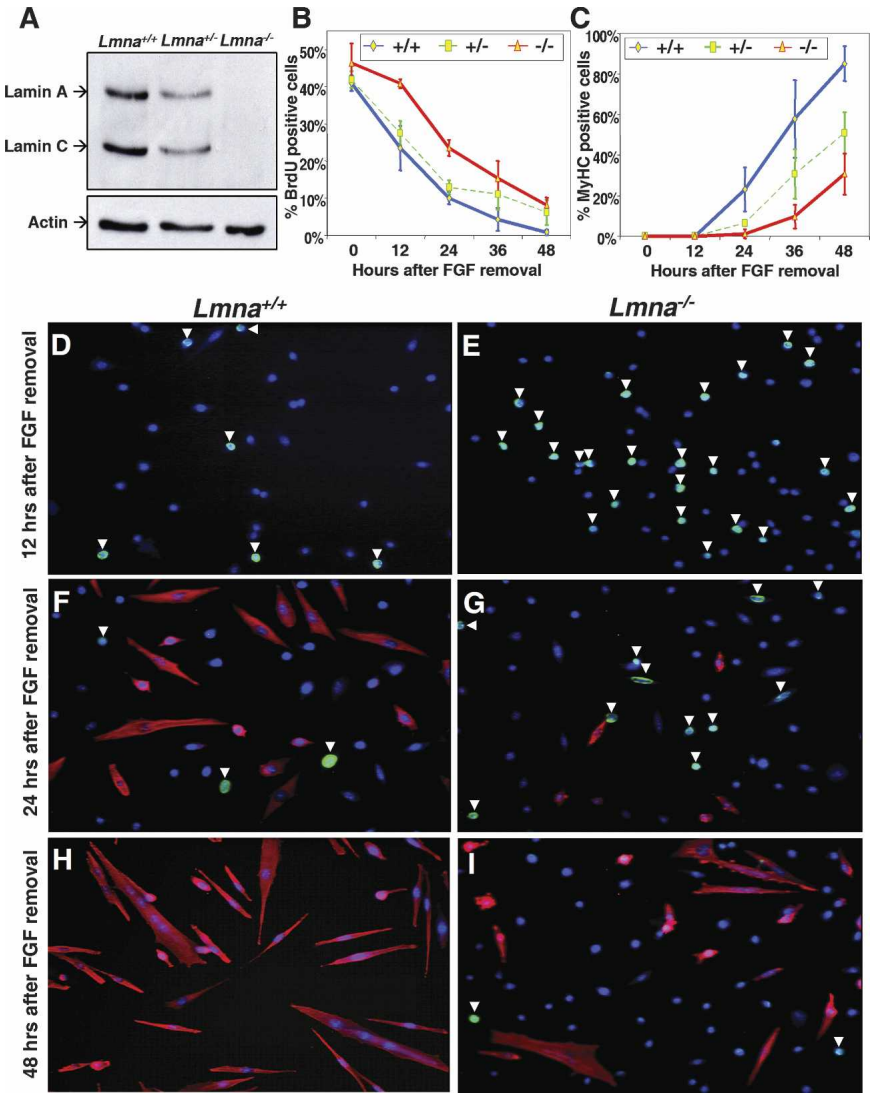

Figure 1. *Lmna*$^{-/-}$ muscle cells have delayed differentiation kinetics. (*A*) Western analysis indicating the lamin A/C proteins present in the permanent *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ myoblast cell lines. (*B*) Fraction of S-phase cells following bFGF removal as measured by cells staining positively for BrdU incorporation in *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ cultures. After 12 h of bFGF deprivation, 40% of *Lmna*$^{-/-}$ muscle cells remain in S phase compared with ~20% of *Lmna*$^{+/+}$ cells. (*C*) The appearance of terminally differentiated muscle protein as measured by cells staining positively for MyHC in identical bFGF-deprived cultures. By 48 h, only ~30% of *Lmna*$^{-/-}$ cells were MyHC-positive compared with ~90% in *Lmna*$^{+/+}$ cells. *Lmna*$^{+/-}$ muscle cells display an intermediate differentiation phenotype relative to *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ muscle cells. Cell counts are based on three experiments with ~350 cells counted per time point. (*D–I*) Immunostained *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ muscle cells at 12 h (*D*,*E*), 24 h (*F*,*G*), and 48 h (*H*,*I*) after mitogen withdrawal. BrdU is in green with arrowheads indicating BrdU-positive cells, MyHC is in red, and Dapi is in blue.

kinetics of skeletal muscle differentiation after bFGF removal can thus be approximated by determining the percentage of cells in S phase by BrdU incorporation and the percentage of cells that exhibit immuno-positive staining for MyHC.

The timing of myogenic terminal differentiation was compared among *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ muscle cells. Initially, 40%–50% of the myoblasts of each genotype are in S phase; this is consistent with the predicted 42% S-phase cells in cultures with a 19-h cell cycle and a typical 8-h S phase (Clegg et al. 1987). After 12 h >40% of *Lmna*$^{-/-}$ myoblasts remain in S phase compared with ~25% of *Lmna*$^{+/+}$ cells (Fig. 1B,D,E); and differences in proliferation still remain after 24 h. These findings indicate that myoblasts with reduced lamin A/C are delayed in their ability to exit the cell cycle in response to mitogen deprivation.

Consistent with the delay in cell cycle withdrawal, the appearance of MyHC-positive cells is delayed ~24 h in *Lmna*$^{-/-}$ compared with *Lmna*$^{+/+}$ cells (Fig. 1C,F,G). Forty-eight hours after bFGF removal, only ~30% of *Lmna*$^{-/-}$ cells were MyHC-positive compared with ~85% of *Lmna*$^{+/+}$ cells (Fig. 1C,H,I). These data indicate that upon growth factor withdrawal, *Lmna*$^{-/-}$ muscle cells are also slower to exhibit the gene expression properties of differentiated muscle. *Lmna*$^{+/-}$ muscle cells are likewise compromised, exhibiting an intermediate differentiation phenotype between that of *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ cells.

The slower differentiation kinetics of *Lmna*$^{-/-}$ muscle cells could be explained by a lag in responsiveness to FGF removal after which, given sufficient time, a majority of cells become myocytes; alternatively, the *Lmna*$^{-/-}$ population may contain some cells capable of normal differentiation and other cells that are unable to terminally differentiate. These possibilities were analyzed by clonal assays in which colonies were grown in bFGF-containing media for 5 d then switched to bFGF-free media and the relative differentiation capacity of individual clones from each cell line was assessed after increasing periods of mitogen deprivation. The extent of differentiation was assessed by determining the percentage of

nuclei in MyHC-positive cells. Individual colonies were then subdivided into four arbitrary categories: 0% MyHC$^+$, <30% MyHC$^+$, 30%–60% MyHC$^+$, and 60%–95% MyHC$^+$, and the data were plotted for clones fixed 4–10 d following mitogen removal (Fig. 2A). In *Lmna*$^{+/+}$ cultures, >95% of the total colonies contained MyHC$^+$ cells by day 4, while *Lmna*$^{-/-}$ and *Lmna*$^{+/-}$ cultures contained 10%–20% fewer MyHC$^+$ colonies (Fig. 2B); and whereas ~80% of the *Lmna*$^{+/+}$ colonies were highly differentiated after 4 d, no *Lmna*$^{-/-}$ clones and only ~25% of the *Lmna*$^{+/-}$ clones were highly differentiated. Significantly, as the post-mitogen withdrawal period increased, <3% of the total *Lmna*$^{-/-}$ colonies ever became highly differentiated, only ~20% ever acquired an intermediate (30%–60% MyHC$^+$) level of differentiation, and the fraction of colonies exhibiting low differentiation levels (<30% MyHC$^+$ cells) increased only slightly. *Lmna*$^{+/-}$ clones, while more differentiated than *Lmna*$^{-/-}$ clones, also exhibited only marginal increases in differentiation at longer periods of mitogen withdrawal. These results are consistent with the hypothesis that most *Lmna*$^{-/-}$ myoblasts are compromised with respect to their differentiation potential. (Evidence that the impaired differentiation phenotype of most *Lmna*$^{-/-}$ and some *Lmna*$^{+/-}$ clones is not due to the common "differentiation-defective" phenotype observed in many permanent mouse and rat myogenic cell lines [Lim and Hauschka 1984] is provided below.)

Lmna$^{-/-}$ *myoblasts exhibit both altered and normal levels of transcription factors and other key myogenic components*

Since differences between *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ muscle cell differentiation are apparent as early as 12 h after bFGF removal, the compromised *Lmna*$^{-/-}$ differentiation phenotype could be due to altered levels or activity of critical regulatory factors in myoblasts prior to the initiation of terminal differentiation. For example, myoblasts from *MyoD*$^{-/-}$ mice also exhibit delayed cell cycle withdrawal kinetics when deprived of growth factors (Sabourin et al. 1999). This hypothesis was examined via Western blot analysis of proliferating *Lmna*$^{-/-}$ and *Lmna*$^{+/+}$ myoblasts (Fig. 3A). This disclosed that in *Lmna*$^{-/-}$ myoblasts MyoD protein levels were reduced by >60% while the levels of Myf-5, a related basic helix–loop–helix (bHLH) transcription factor whose expression is often up-regulated in response to decreased MyoD levels (Rudnicki et al. 1992), were highly increased. Several studies have demonstrated that pRB acts synergistically with MyoD to activate transcription of muscle specific factors (Puri et al. 2001; Guo et al. 2003), and that pRB is also required for cell cycle exit accompanying muscle differentiation (Novitch et al. 1996, 1999; Huh et al. 2004). pRB Western blot analysis indicated that steady-state levels of both hyper- and hypo-phosphorylated pRB were dramatically reduced in *Lmna*$^{-/-}$ myo-

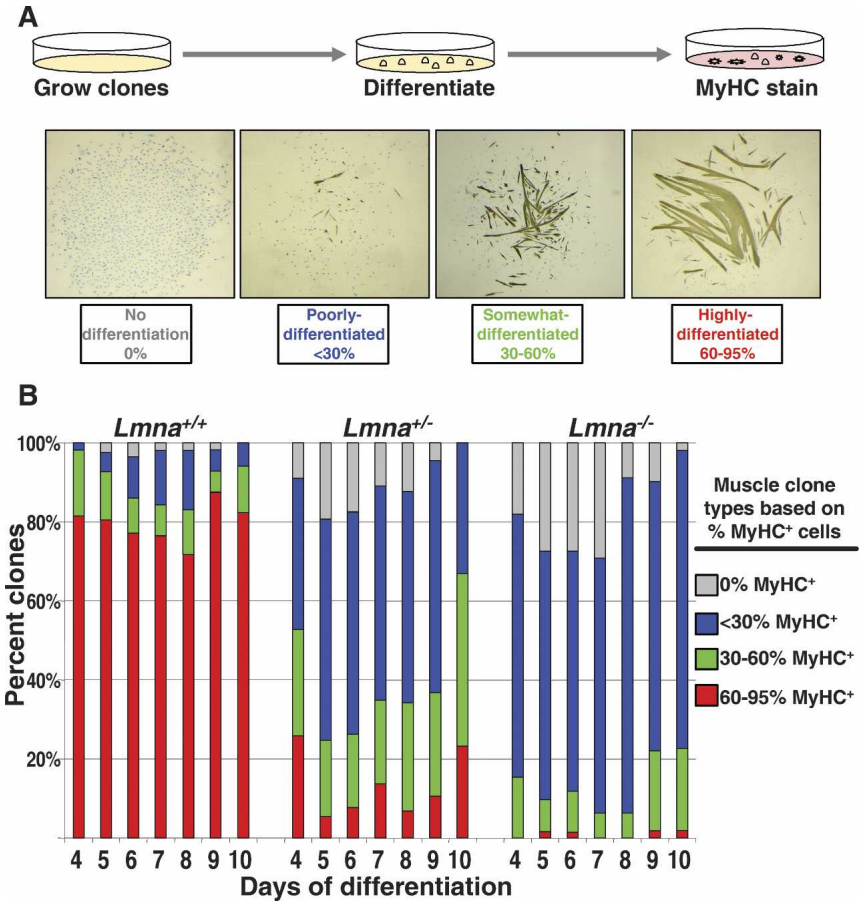

Figure 2. *Lmna*$^{-/-}$ muscle cells have reduced differentiation potential. (*A*) Method for the clonal analysis of differentiation: Cells are plated at low cell densities and given 5 d to form colonies, then switched to FGF-free, low-serum media to induce differentiation. The relative differentiation capacity of individual clones from each cell line was measured at successive days of mitogen deprivation. Fixed cultures were immunostained for MyHC (brown) and counterstained for total cells with 1% methylene blue (light blue). Clones were then subdivided into four arbitrary categories based on percentage of nuclei in MyHC$^+$ cells: 0%, <30%, 30%–60%, and 60%–95%. (*B*) Clonal analysis of differentiation for *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ muscle cells from 4 to 10 d of differentiation. Approximately 80% of clones in *Lmna*$^{+/+}$ cultures were highly differentiated at day 4, whereas none of the *Lmna*$^{-/-}$ colonies were similarly differentiated. As the post-mitogen withdrawal period increased, <3% of the *Lmna*$^{-/-}$ colonies ever became highly differentiated. Consistent with mass culture differentiation (see Fig. 1C), *Lmna*$^{+/-}$ muscle cells display an intermediate differentiation phenotype relative to *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ muscle cells. Approximately 100 clones were scored per time point.

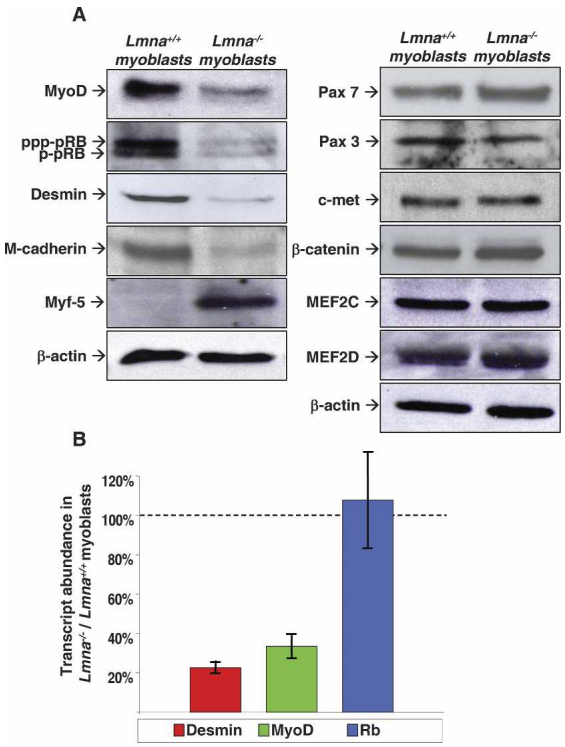

Figure 3. MyoD, pRB, desmin, and M-cadherin proteins are reduced in proliferating *Lmna*$^{-/-}$ myoblasts. (*A*) Western analysis comparing relative protein levels of myogenic factors in *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts. MyoD, pRB, desmin, and M-cadherin proteins are reduced, whereas Myf-5 protein levels are increased in *Lmna*$^{-/-}$ myoblasts. In addition, Pax7, Pax3, MEF2C, MEF2D, c-met, and β-catenin proteins are unaffected in *Lmna*$^{-/-}$ myoblasts. (*B*) QPCR analysis comparing the relative mRNA's of desmin, MyoD, and pRB in *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts. Desmin and MyoD mRNA's are 4.5-fold and threefold reduced in *Lmna*$^{-/-}$ myoblasts, whereas *Rb* mRNA is not affected. Data represent averages of triplicate experiments performed at three different dilutions of cDNA. Fold changes were measured by comparing Ct values from *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblast mRNAs that were normalized against *Hprt*.

blasts compared with *Lmna*$^{+/+}$ controls (Fig. 3A). In contrast to the MyoD, Myf-5, and pRB changes, the protein levels of several other key myogenic transcription factors: MEF2C, MEF2D, Pax3, and Pax7 were unperturbed in proliferating *Lmna*$^{-/-}$ myoblasts.

It also seemed informative to determine whether *Lmna*$^{-/-}$ myoblasts exhibited altered levels of important myogenic structural and signaling proteins. The muscle-specific cytoplasmic intermediate filament protein desmin was analyzed in *Lmna*$^{-/-}$ myoblasts for several reasons. First, desmin is required for normal differentiation in vitro (Li et al. 1994; Weitzer et al. 1995), and desmin knockout myoblasts also have prolonged cell cycle withdrawal kinetics and delayed fusion in vivo (Smythe et al. 2001). Second, *MyoD*$^{-/-}$ primary mouse myoblasts do not express desmin (Sabourin et al. 1999). Third, EM studies have found abnormal desmin localization in *Lmna*$^{-/-}$ cardiomyocytes (Nikolova et al. 2004). Interestingly, desmin protein levels were significantly reduced in proliferating *Lmna*$^{-/-}$ myoblasts (Fig. 3A). Because reduced desmin levels might have resulted in compensation by other cytoplasmic intermediate filament proteins such as vimentin and nestin, the levels of these proteins were also examined in *Lmna*$^{-/-}$ myoblasts. However, vimentin and nestin occur at normal levels in myoblasts lacking lamin A/C (data not shown).

The Ca^{+}-dependent cell adhesion protein M-cadherin was analyzed because its gene is activated by MyoD (Sabourin et al. 1999; Cornelison et al. 2000) and because M-cadherin plays roles in cell fusion and other aspects of myogenesis (Zeschnigk et al. 1995; Kang et al. 2003). M-cadherin protein levels were also significantly reduced in *Lmna*$^{-/-}$ myoblasts relative to *Lmna*$^{+/+}$ myoblasts (Fig. 3A). The tyrosine kinase receptor c-met was analyzed because its overexpression is known to inhibit myogenic differentiation (Anastasi et al. 1997), and β-catenin was analyzed because it is known to play multiple roles during myogenesis (Petropoulos and Skerjanc 2002), but neither protein exhibited altered levels in *Lmna*$^{-/-}$ myoblasts (Fig. 3A). Taken together, these results indicate that myoblasts lacking lamin A/C exhibit several major differences in regulatory, structural, and signaling components, one or more of which could potentially account for the compromised differentiation of *Lmna*$^{-/-}$ myoblasts in response to growth factor deprivation.

MyoD and desmin, but not Rb, transcripts, are reduced in Lmna$^{-/-}$ *myoblasts*

Why are the levels of MyoD, desmin, and pRB proteins reduced in proliferating *Lmna*$^{-/-}$ myoblasts? Quantitative PCR (QPCR) was used to determine whether reduced levels of the three proteins correlated with changes in transcript levels. *MyoD* and *Des* transcript levels were reduced by more than threefold and 4.5-fold, respectively, in the *Lmna*$^{-/-}$ myoblasts (Fig. 3B). In contrast, *Rb* transcript levels were unaffected in *Lmna*$^{-/-}$ myoblasts relative to *Lmna*$^{+/+}$ myoblasts (Fig. 3B). We posit that pRB protein stability is reduced in these cells, perhaps through enhanced proteasome-dependent degradation, as has been observed in *Lmna*$^{-/-}$ fibroblasts (Johnson et al. 2004).

Individual Lmna$^{-/-}$ *myoblasts express variable levels of MyoD and desmin, but normal levels of Pax7*

Clonal analysis of *Lmna*$^{-/-}$ myoblasts indicates that fewer cells in most colonies retain the capacity to differentiate rapidly upon bFGF removal, and most cells fail to differentiate even after long time periods. We considered several explanations for these differences with respect to MyoD, desmin, and Pax7 levels. First, there may be several subpopulations of *Lmna*$^{-/-}$ cells: one with near-normal levels of proteins required for the myogenic program, which retain a full differentiation potential, and others with variably decreased expression of MyoD and/or desmin that have lower probabilities of differentiation. Alternatively, one or more of these proteins may be reduced in all cells, again leading to lower probabilities of differentiation. To distinguish between these possi-

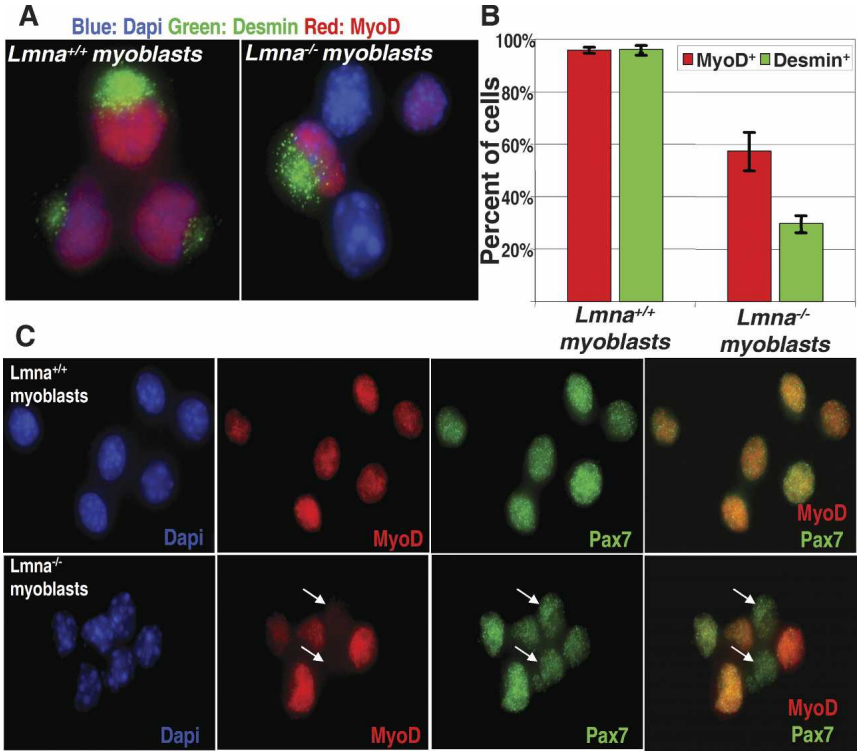

Figure 4. *Lmna*$^{-/-}$ myoblasts exhibit mixed heterogeneity with respect to MyoD and desmin expression. (*A*) Immunostaining of *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts using antibodies to MyoD (red) and desmin (green). Images are a two-dimensional representation of serial images taken throughout the cells (volume view). When present, MyoD and Pax7 occupy the entire nucleus, whereas desmin is localized to a portion of the cytoplasm. (*B*) Graph summarizing cell counts of MyoD and desmin immunopositive cells in *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts. Only ~30% of *Lmna*$^{-/-}$ myoblasts are desmin-positive and ~57% of *Lmna*$^{-/-}$ myoblasts are MyoD-positive. Data represent average from three different experiments, ~400 cells/experiment. (*C*) Indirect immunofluorescence of *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts using antibodies to MyoD (red) and Pax7 (green) with Dapi (blue) for total cells. Arrows indicate MyoD-negative cells that are Pax7-positive. As in *A*, images are presented as a volume view.

bilities, single *Lmna*$^{-/-}$ myoblasts were immunostained using antibodies to either desmin or MyoD (Fig. 4A). About 70% of *Lmna*$^{-/-}$ myoblasts exhibit low or undetectable desmin immunofluorescence, whereas 30% exhibit relatively normal staining (Fig. 4B). In *Lmna*$^{+/+}$ control myoblasts, only a small percentage (~4%) exhibited low or undetectable desmin staining. MyoD staining was also reduced in *Lmna*$^{-/-}$ cells and again was nonuniformly distributed in individual cells (Fig. 4A). In this case, however, a higher percentage of *Lmna*$^{-/-}$ myoblasts (~57%) retained a detectable signal (Fig. 4B). To address the myogenic status of the MyoD-negative *Lmna*$^{-/-}$ myoblasts, cells were immunostained for Pax7 protein. Consistent with Western analysis (Fig. 3A), both *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts are highly Pax7-positive. Furthermore, 96% (144/150 cells) of the MyoD-negative *Lmna*$^{-/-}$ myoblasts are also Pax7-positive (Fig. 4C), thus suggesting most MyoD-negative-*Lmna*$^{-/-}$ myoblasts retain some myogenic identity (Relaix et al. 2005).

RNA interference (RNAi)-mediated silencing of Lmna *impairs differentiation and leads to reduced MyoD and desmin protein levels*

To eliminate the possibility that the phenotypes of *Lmna*$^{-/-}$ myoblasts were due to inadvertent cell culture selection for differentiation-defective muscle cells, we tested whether the reduced differentiation potential of *Lmna*$^{-/-}$ myoblasts could be phenocopied by reducing lamin A/C levels via siRNA targeting of the *Lmna* mRNAs (Brummelkamp et al. 2002). *Lmna*$^{+/+}$ myoblasts were stably transduced with retroviruses expressing short hairpin RNAs targeted to the *Lmna* transcript, and control *Lmna*$^{+/+}$ myoblasts were transduced with RNAi targeted to EGFP (see Materials and Methods). Lamin A/C protein levels were reduced ~10-fold under these conditions (Fig. 5A). It should be noted that these cells express almost no detectable lamin C since the RNAi targets endogenous mRNAs encoding both lamin A and lamin C. To test their ability to differentiate, both the EGFP-RNAi *Lmna*$^{+/+}$ and *Lmna*-RNAi *Lmna*$^{+/+}$ muscle cells were subjected to myogenic clonal analysis. Importantly, the clonal assays were initiated within 4 d of the siRNA transduction, thereby eliminating the possibility of gradual in vitro selection for differentiation-defective myoblasts. The expression of the EGFP-RNAi in *Lmna*$^{+/+}$ myoblasts had no effect on cell differentiation. In contrast, as with *Lmna*$^{-/-}$ myoblasts, *Lmna*-RNAi *Lmna*$^{+/+}$ muscle cells produced fewer well-differentiated clones than the control EGFP-RNAi *Lmna*$^{+/+}$ muscle cells (Fig. 5C). Also consistent with the phenotype of *Lmna*$^{-/-}$ myoblasts, protein levels of both MyoD and desmin are reduced in *Lmna*$^{+/+}$ myoblasts expressing the *Lmna*-RNAi (Fig. 5B). However, the protein levels of pRB are only slightly decreased in *Lmna*-RNAi *Lmna*$^{+/+}$ myoblasts (Fig. 5B). Therefore, the phenotypes of *Lmna*$^{-/-}$ muscle cells are generally, but not completely, recapitulated by reducing lamin A/C expression in *Lmna*$^{+/+}$ cells.

Expression of lamin A rescues muscle differentiation in Lmna*-silenced* Lmna$^{+/+}$ *myoblasts but not in* Lmna$^{-/-}$ *myoblasts*

To confirm that defects in myogenic differentiation observed in myoblasts with reduced lamin A/C levels are

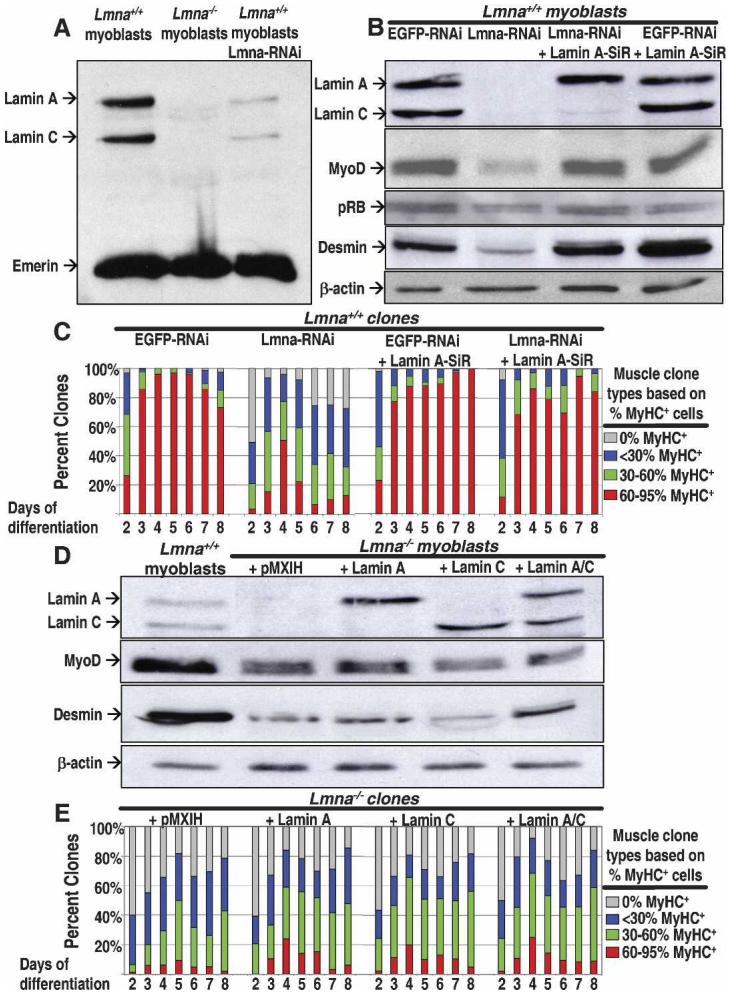

Figure 5. Analysis of *Lmna*$^{-/-}$ and *Lmna*-RNAi-treated *Lmna*$^{+/+}$ muscle cultures with and without the addition of lamin A. (*A*) Western analysis of *Lmna*$^{+/+}$ myoblasts indicating ~10-fold reduction in lamin A/C protein levels in the presence of retrovirally transduced *Lmna*-RNAi. (*B*) *Lmna*$^{+/+}$ myoblasts retrovirally transduced with EGFP-RNAi alone, *Lmna*-RNAi alone, *Lmna*-RNAi + lamin A-SiR, or EGFP-RNAi + lamin A-SiR were probed for relative amounts of MyoD, pRB, and desmin proteins. Protein levels of MyoD and desmin are reduced in *Lmna*$^{+/+}$ myoblasts expressing the *Lmna*-RNAi, but can be recovered when lamin A-SiR is subsequently expressed in the same cells. Protein levels of pRB are slightly reduced when *Lmna*-RNAi is expressed. (*C*) Myogenic clonal analysis of *Lmna*$^{+/+}$ muscle cells retrovirally transduced with EGFP-RNAi alone, *Lmna*-RNAi alone, EGFP-RNAi + lamin A-SiR, or *Lmna*-RNA + lamin A-SiR. Reducing lamin A/C protein levels results in fewer clones that are highly differentiated compared with the EGFP-RNAi control; increasing lamin A protein levels in *Lmna*-RNAi *Lmna*$^{+/+}$ muscle cells restores differentiation potential. Approximately 150 clones were scored per time point. (*D*) Retroviral transduction of lamin A, lamin C, or lamin A/C in *Lmna*$^{-/-}$ myoblasts does not restore MyoD or desmin proteins to wild-type levels. (*E*) Differentiation potential of *Lmna*$^{-/-}$ muscle cells is not significantly increased when lamin A, lamin C, or both are expressed. Approximately 150 clones were scored per time point.

indeed specific to the loss of lamin, we attempted to rescue the differentiation defects by stable reintroduction of lamin A. So that the attempted phenotypic rescue of *Lmna*$^{-/-}$ myoblasts could be compared with rescue of *Lmna*-silenced *Lmna*$^{+/+}$ myoblasts, the lamin A "rescue cDNA" was designed to contain several silent codon changes at the target site of the siRNA, thereby rendering it resistant to the siRNA (lamin A-SiR; see Materials and Methods). *Lmna*$^{+/+}$ myoblasts that had been stably transduced with either EGFP-RNAi or *Lmna*-RNAi were subjected to a second round of retroviral transduction and selection for stably transduced myoblasts that express lamin A-SiR (Fig. 5B). Analysis of the *Lmna*-RNAi-transduced myoblasts indicates that the protein levels of MyoD and desmin are restored to wild-type levels (Fig. 5B) and that they differentiate normally (Fig. 5C), consistent with the hypothesis that the defective differentiation of *Lmna*-silenced *Lmna*$^{+/+}$ myoblasts is indeed a direct result of reduced lamin A/C expression. However, attempts to obtain the same phenotypic rescue by expressing lamin A in *Lmna*$^{-/-}$ myoblasts have been unsuccessful. Such cells do not exhibit increased differentiation potential relative to control *Lmna*$^{-/-}$ cells transduced with either lamin A, lamin C, or both (Fig. 5E), nor are the protein levels of MyoD and desmin restored to wild-type levels (Fig. 5D). Possible reasons for this are addressed in the Discussion.

Exogenous expression of desmin or MyoD in Lmna$^{-/-}$ *myoblasts increases differentiation potential*

Previous studies have demonstrated that a loss or reduction of desmin results in impaired muscle differentiation (Li et al. 1994; Weitzer et al. 1995; Smythe et al. 2001). Since desmin levels are reduced in myoblasts lacking lamin A/C, we hypothesized that their differentiation potential might be restored by exogenous expression of desmin. Thus, stably transduced *Lmna*$^{-/-}$ myoblast lines were created that express desmin by using a retroviral approach similar to that described for lamin A (Fig. 6A). These *Lmna*$^{-/-}$ myoblasts exhibited near normal desmin protein levels, but their MyoD levels remained low (Fig. 6A); yet despite their reduced MyoD, the percentage of well differentiated muscle colonies was two to three times greater than that of *Lmna*$^{-/-}$ myoblasts transduced with a control expression vector (Fig. 6B).

The reduction of both MyoD and desmin mRNAs in *Lmna*$^{-/-}$ muscle cells suggests that lamin A/C function

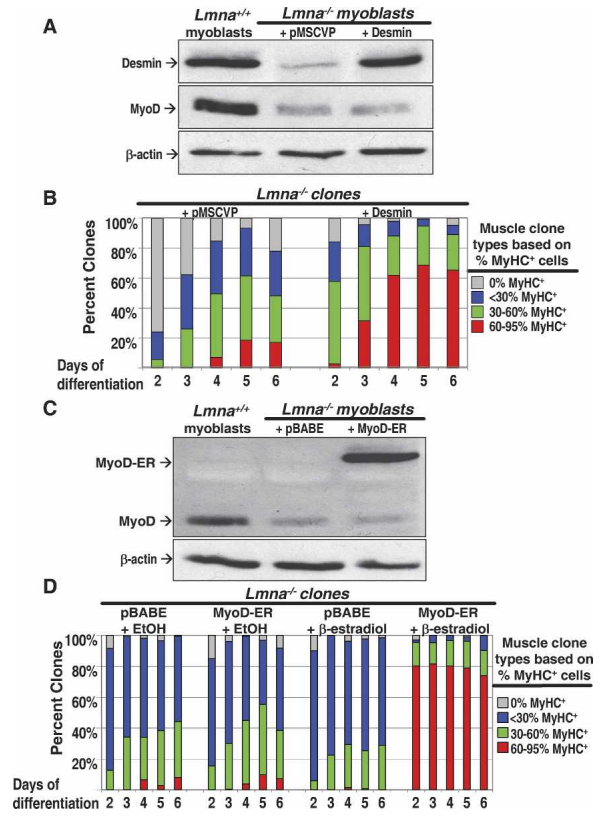

Figure 6. Forced expression of desmin or MyoD in *Lmna*$^{-/-}$ myoblasts increases their differentiation potential. (*A*) Western blot showing that forced expression of retrovirally transduced desmin to near wild-type protein levels in *Lmna*$^{-/-}$ myoblasts does not increase MyoD protein levels. (*B*) Myogenic clonal analysis comparing *Lmna*$^{-/-}$ muscle cells expressing either vector control (pMSCVP) or desmin. Forced expression of desmin enhances the differentiation potential of *Lmna*$^{-/-}$ muscle cells to near wild-type levels. Approximately 250 clones were scored per time point. (*C*) Western analysis comparing forced expression of retrovirally transduced MyoD-ER fusion protein in *Lmna*$^{-/-}$ myoblasts to MyoD in *Lmna*$^{+/+}$ myoblasts. (*D*) Clonal analysis indicates that estradiol treatment of MyoD-ER-expressing *Lmna*$^{-/-}$ muscle cells causes ~80 percent of clones to become highly differentiated, whereas control cultures (pBABE ± β-estradiol and MyoD-ER + vehicle) exhibit few if any highly differentiated muscle clones. Approximately 150 clones were scored per time point.

might be important for efficient transcription of either or both these genes. MyoD has been shown to enhance desmin expression in vitro (Li and Capetanaki 1993), but this activity is reported to be specific for differentiating muscle cells, not proliferating myoblasts (Gao et al. 1998). To determine whether elevated MyoD expression in *Lmna*$^{-/-}$ myoblasts would rescue their compromised differentiation, cells were transduced with MyoD or control retroviral vectors. Since exogenous expression of MyoD activates the muscle differentiation program in a variety of cells (Tapscott et al. 1988), we used a conditional MyoD-estrogen receptor (ER) system in which MyoD-ER accumulates in the cytoplasm until the cells are exposed to β-estradiol, which upon binding the ER moiety, targets MyoD-ER to the nucleus (Hollenberg et al. 1993). Stably transduced control and MyoD-ER-*Lmna*$^{-/-}$ myoblasts (Fig. 6C) were plated at clonal densities, grown for 5 d, switched to FGF-free medium in the presence or either β-estradiol or vehicle control (+EtOH), and then periodically fixed and immunostained for MyHC (Fig. 6D). MyoD-ER-*Lmna*$^{-/-}$ colonies exposed to β-estradiol exhibited high levels of differentiation within 2 d, whereas three different sets of control colonies exhibited delayed and less extensive differentiation. Importantly, the differentiation of β-estradiol-treated MyoD-ER-*Lmna*$^{-/-}$ colonies was greater than that of desmin-*Lmna*$^{-/-}$ myoblasts (Fig. 6B) and equivalent to that of *Lmna*$^{+/+}$ muscle cells (Fig. 2B).

To further investigate differences between the differentiation kinetics of MyoD-ER-*Lmna*$^{-/-}$ myoblasts and desmin-*Lmna*$^{-/-}$ myoblasts, both cell types were assayed in mass cultures during the first 48 h after FGF removal. MyoD-ER-*Lmna*$^{-/-}$ cells exhibited rapid cell cycle withdrawal and >95% of the cells were MyHC-positive within 48 h, while desmin-*Lmna*$^{-/-}$ myoblasts exhibited virtually no cell cycle withdrawal during the first 12 h and significantly lower levels of differentiation by 48 h (Table 1, red numerals; also cf. Fig. 1B,C). These findings suggest that unlike readdition of MyoD-ER, which enhances both the timing of cell cycle exit and the differentiation potential of *Lmna*$^{-/-}$ cells to wild-type levels, readdition of desmin is not sufficient to restore the normal kinetics of cell cycle exit after FGF-withdrawal of *Lmna*$^{-/-}$ cells, but constitutively expressed desmin improves myogenesis after these cells eventually withdraw from the cell cycle. Collectively, these findings indicate that the compromised differentiation potential of myoblasts lacking lamin A/C may derive from reduced levels of desmin and MyoD, and the restoration of each protein can at least partially correct the compromised levels of myogenic differentiation.

Reduced expression of Emd leads to defects in muscle differentiation

Mutations in a second gene, *EMD*, encoding the lamin-associated protein emerin have been identified in EDMD1. Since emerin and lamin A/C are known to interact within the nuclear envelope (Gruenbaum et al. 2005), we tested whether forced reduction in emerin expression would lead to defects in muscle differentiation similar to those exhibited by lamin A/C silenced cells. An RNAi specific to emerin was created and introduced in *Lmna*$^{+/+}$ myoblasts using the same retroviral approach described previously for *Lmna*-RNAi. Expression of *Emd*-RNAi led to near total elimination of emerin protein (Fig. 7A). When these muscle cells were subjected to myogenic clonal analysis, <50% of the clones scored as highly differentiated (Fig. 7B). In addition, the protein levels of MyoD and desmin, but not pRB, are reduced to levels similar to *Lmna*-RNAi-treated cells (Fig. 5B). Since the levels of MyoD were reduced in *Emd*-RNAi *Lmna*$^{+/+}$ myoblasts, we hypothesized that forced expres-

Table 1. *Forced expression of MyoD, but not desmin, rescues cell cycle withdrawal and early differentiation kinetics in* Lmna$^{-/-}$ *cells*[a]

A. CELL CYCLE WITHDRAWAL:	Percent *Lmna*$^{-/-}$ cells positive for BrdU[b]					
Differentiation Condition:	(Vehicle[c])		(0.1μM β-estradiol)		(None)	
Hours after FGF removal	Vector	MyoD-ER	Vector	MyoD-ER	Vector	Desmin
0 hours	51.2 ± 0.4	51.9 ± 2.7	51.2 ± 0.4	51.9 ± 2.7	54.6 ± 2.9	51.2 ± 3.2
12 hours	43.6 ± 1.4	38.2 ± 3.9	42.6 ± 2.9	7.8 ± 2.9[d]	48.8 ± 4.9	45.0 ± 3.1[f]
48 hours	6.8 ± 0.7	4.5 ± 1.5	7.4 ± 2.2	2.8 ± 0.4	9.6 ± 4.7	8.1 ± 2.3

B. DIFFERENTIATION:	Percent *Lmna*$^{-/-}$ cells positive for MyHC[b]					
Differentiation Condition:	(Vehicle[c])		(0.1μM β-estradiol)		(None)	
Hours after FGF removal	Vector	MyoD-ER	Vector	MyoD-ER	Vector	Desmin
0 hours	0.0	0.0	0.0	0.0	0.0	0.0
12 hours	0.7 ± 0.6	0.7 ± 0.3	1.7 ± 1.1	0.4 ± 0.4	0.1 ± 0.2	0.1 ± 0.1
48 hours	35.7 ± 1.3	41.2 ± 6.6	31.0 ± 3.0	95.9 ± 1.4[e]	34.1 ± 6.5	30.6 ± 8.9[f]

[a]Black and blue text indicates cell cycle withdrawal and differentiation subpanels respectively. Red text highlights key points in the table.
[b]Based on average and SD of three experiments counting ~650 cells/time point/experiment in mass culture.
[c]Vehicle for solubilizing β-estradiol consists of 10 μL 100% EtOH in 10 mL of media.
[d]Estradiol-mediated nuclear import of MyoD-ER accelerates cell cycle withdrawal of *Lmna*$^{-/-}$ cells following FGF deprivation.
[e]Estradiol-mediated nuclear import of MyoD-ER greatly enhances the proportion of terminally differentiated *Lmna*$^{-/-}$ cells.
[f]Overexpression of desmin has no cell cycle withdrawal or early differentiation effects on *Lmna*$^{-/-}$ cells.

sion of MyoD using the MyoD-ER system described above would restore their differentiation potential. Stably transduced control and MyoD-ER-*Emd*-RNAi *Lmna*$^{+/+}$ myoblasts (Fig. 7C) were plated at clonal densities, grown for 5 d, switched to FGF-free medium in the presence of either β-estradiol or vehicle control (+EtOH), and then periodically fixed and immunostained for MyHC (Fig. 7D). Similarly to MyoD-ER-*Lmna*$^{-/-}$ myoblasts, the differentiation of β-estradiol-treated MyoD-ER-*Emd*-RNAi *Lmna*$^{+/+}$ colonies was greater than that of three different sets of control colonies (Fig. 7D) and similar to that of *Lmna*$^{+/+}$ muscle cells (see Fig. 2B). These data suggest that readdition of MyoD in *Emd*-RNAi *Lmna*$^{+/+}$ myoblasts can restore differentiation potential similar to that of *Lmna*$^{+/+}$ myoblasts. Therefore, myoblasts with either reduced lamin A/C levels or reduced emerin levels display delayed differentiation kinetics and have reduced levels of a subset of proteins important for myogenic differentiation.

Discussion

Mutations in the genes encoding the nuclear proteins emerin and lamin A/C are associated with EDMD. Although their roles in skeletal muscle development are unknown, we demonstrate that reducing lamin A/C or emerin via *Lmna* knockout, *Lmna*-RNAi, and *Emd*-RNAi results in reduced levels of desmin and MyoD in myoblasts, and that each of the independent perturbations causes a similar set of compromised differentiation phenotypes. Restoration of MyoD, and more surprisingly desmin, significantly increases the differentiation potential of lamin A/C-deficient myoblasts, suggesting that reduced levels of these proteins may underlie the differentiation impairment of *Lmna*$^{-/-}$ muscle cells.

Differentiation defects in myoblasts lacking A-type lamins or emerin

The reduction in desmin and MyoD transcripts in *Lmna*$^{-/-}$ myoblasts suggests that A-type lamins are directly or indirectly affecting the transcription of both genes. Previous studies indicated that desmin is a target gene for MyoD, although MyoD dependence was reported to be important for proper levels of desmin transcription only *after* induction of terminal differentiation (Gao et al. 1998). However, our studies indicate a 4.5-fold reduction of desmin mRNA in *Lmna*$^{-/-}$ myoblasts. Does the reduction or absence of A-type lamins have nonspecific global effects on the transcription of many genes, some of which happen to play important roles in muscle differentiation, or does lamin A/C affect specific gene targets important for maintaining the myogenic determination of skeletal muscle cells? A-type lamins interact with and organize chromatin (Glass et al. 1993) in a manner possibly mediated through lamin–histone interactions (Taniura et al. 1995). Therefore, loss or reduction of lamin A/C could affect the expression of numerous genes. In addition, A-type lamins have been shown to directly interact with transcription factors such as pRB (Ozaki et al. 1994; Markiewicz et al. 2002), SREBP-1 (Lloyd et al. 2002), and MOK2 (Dreuillet et al. 2002), implying that they could also act as accessory proteins for a subset of tissue-defining factors. Our data show that although desmin, MyoD, pRB, and M-cadherin are reduced and Myf-5 is increased in the absence of A-type lamins, other myogenic factors such as Pax3, Pax7, MEF2C, MEF2D, c-met, and β-catenin are not affected, indicating that A-type lamins have a specific effect on a subset of myogenic components. While the consequence of decreased pRB and M-cadherin protein levels remains to be determined, it seems unlikely that decreased pRB

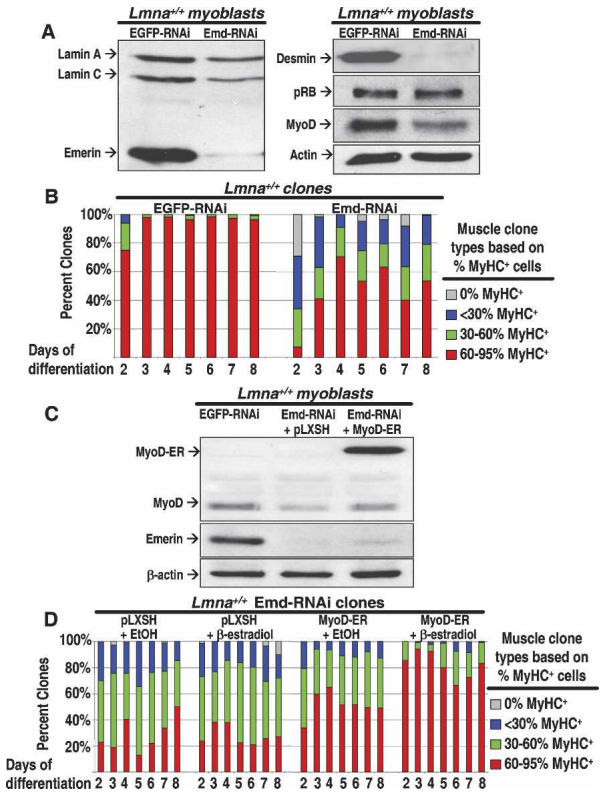

Figure 7. Reducing protein levels of emerin impairs muscle differentiation. (*A*) *Lmna*$^{+/+}$ myoblasts with retrovirally transduced *Emd*-RNAi exhibit greatly reduced emerin protein as well as reductions in MyoD and desmin, but not pRB. (*B*) Clonal analysis indicates that few highly differentiated muscle clones are formed by *Lmna*$^{+/+}$ muscle cells expressing *Emd*-RNAi compared with *Lmna*$^{+/+}$ EGFP-RNAi control muscle cells. Approximately 120 clones were scored per time point. (*C*) Western analysis indicating the expression of MyoD-ER in *Lmna*$^{+/+}$ *Emd*-RNAi myoblasts. (*D*) Clonal analysis of *Lmna*$^{+/+}$ Emd-RNAi cells. Increased numbers of well-differentiated clones appear when MyoD-ER is expressed, and cells are differentiated in the presence of β-estradiol. Approximately 100 clones were scored per time point.

levels alone underlie the common differentiation defects seen in response to lamin A/C deficiencies, because pRB levels are not reduced in response to either *Lmna*- or *Emd*-RNAi yet differentiation is compromised. That pRB may, however, play a role in the reduced differentiation of *Lmna*$^{-/-}$ myoblasts would be compatible with the known interactions of pRB and MyoD with histone deacetylase HDAC1 (Puri et al. 2001). Further evidence consistent with compromised function of pRB and MyoD pathways is provided by recent gene expression profiling studies of EDMDs and characterization of the emerin-null mouse (Bakay et al. 2006; Melcon et al. 2006).

Reintroduction of lamin A protein is sufficient to rescue the differentiation defects in myoblasts with an siRNA-mediated reduction of A-type lamins, but insufficient to rescue differentiation in *Lmna*$^{-/-}$ myoblasts. What underlies this surprising difference? We cannot categorically rule out a model whereby other events have occurred either during the generation of *Lmna*$^{-/-}$ mice or the permanent myoblast cell lines, which further impair in vitro myogenesis. However, we think it unlikely that impaired differentiation kinetics of myoblasts lacking lamin A/C are due to the "differentiation-defective" phenotype that commonly appears in permanent myogenic cell lines (Lim and Hauschka 1984). The strongest evidence against this possibility is that the RNAi-transduced *Lmna*$^{+/+}$ myoblasts were subjected to clonal assays within only 4 d of retroviral transduction, and yet ~50% of the cells generated poorly differentiated clones (<30% MyHC$^+$ cells) while ~95% of the control population generated well-differentiated clones. Since differentiation-defective myoblasts arise at frequencies well below 0.1% and take months of continuous passage to accumulate to substantial numbers (Lim and Hauschka 1984), 50% of the *Lmna*-RNAi population could not have acquired the classic muscle culture differentiation-defective phenotype within 4 d. Additional evidence that the impaired differentiation of *Lmna*$^{-/-}$ myoblasts is not due to the classical differentiation-defective phenotype is that "standard" differentiation-defective muscle cells exhibit normal levels of lamin A/C but do not express Pax 7 (R.L. Frock, S.D. Hauschka, and B.K. Kennedy, unpubl.), whereas *Lmna*$^{-/-}$ myoblasts lack lamin A/C and express Pax7. The low differentiation potential of *Lmna*$^{-/-}$ myoblasts thus seems to result directly from a lamin A/C deficiency.

The individual *Lmna*$^{-/-}$ muscle cells (Fig. 1I) and the small fraction of *Lmna*$^{-/-}$ myogenic clones that exhibit high levels of muscle differentiation (Fig. 2B) raise a paradoxical and as yet unanswered question. Why are some cells able to differentiate while most others are not, when none of the cells contain lamin A/C? The most likely explanation would seem to be that at least one of the molecular processes responsible for committing cells to terminal differentiation is stochastic (Wright 1984), and that lamin A/C levels affect the probability of cell commitment. Clearly, what appears as ostensibly normal muscle differentiation does not require lamin A/C, but the functional levels of A-type lamins appear to influence the commitment probability. In the presence of normal lamin A/C levels, virtually all *Lmna*$^{+/+}$ muscle colonies differentiate; however, the percentage of differentiated cells differs from colony to colony with no colonies containing 100% differentiated cells. Furthermore, the percentage of differentiated cells increases with time (Fig. 1C,D,F,H). Consequently, while overall environmental conditions (e.g., low growth factor levels) may be permissive for differentiation, satellite cell-derived myoblasts have the option of entering additional rounds of DNA synthesis prior to differentiating, and a small percentage become quiescent without differentiating (Angello and Hauschka 1996).

Lmna$^{+/-}$ and *Lmna*-RNAi *Lmna*$^{+/+}$ muscle cells have progressively lower levels of lamin A/C, and they exhibit progressively lower probabilities of differentiating (Fig. 1C). This may be partially attributable to the lower levels of MyoD in such cells (Fig. 3A). However, when individual *Lmna*$^{+/+}$ and *Lmna*$^{-/-}$ myoblasts are immunos-

tained for MyoD (Fig. 4), *Lmna*$^{+/+}$ cells exhibit strong to medium staining intensities, whereas *Lmna*$^{-/-}$ myoblasts exhibit MyoD staining intensities ranging from ~40% that exhibit virtually no staining to many that exhibit staining intensities equivalent to those seen in *Lmna*$^{+/+}$ cells. Importantly, >95% of the MyoD-negative *Lmna*$^{-/-}$ myoblasts are Pax7-positive; thus while these cells exhibit compromised differentiation capabilities, they apparently retain some myogenic properties. While MyoD staining intensities cannot yet be correlated with a cell's subsequent differentiation potential, this heterogeneity could partially explain the compromised differentiation of *Lmna*$^{-/-}$ myoblasts. This possibility would be consistent with the heterogeneous behavior of normal satellite cells in which a low-level commitment to terminally differentiate is correlated with a low ratio of MyoD to Pax7 (Zammit et al. 2004), a phenotype that is also characteristic of some lamin A/C-deficient myoblasts (Fig. 4C,D). Interestingly, when MyoD or desmin are exogenously expressed in lamin A/C-deficient cells, their probability of differentiation returns to near normal levels. Thus MyoD, desmin, and lamin A/C seem to play critical roles in regulating at least one stochastic component of myogenic terminal differentiation.

Desmin and the EDMD phenotype

Previous in vitro cell culture data have suggested a role for desmin in myogenesis (Li et al. 1994; Weitzer et al. 1995). Although *Des*$^{-/-}$ mice develop normally (Li et al. 1996), they have postnatal defects in skeletal, cardiac, and smooth muscles. In addition, *Des*$^{-/-}$ myoblasts have delayed cell cycle withdrawal and delayed fusion in vivo (Agbulut et al. 2001; Smythe et al. 2001), which suggests that desmin is important for adult skeletal muscle regeneration. Our findings with lamin A/C-deficient myoblasts, showing a decrease in desmin protein and transcript, suggest phenotypic overlap between *Lmna*$^{-/-}$ and *Des*$^{-/-}$ mice. In support of this hypothesis, restoration of normal desmin levels in *Lmna*$^{-/-}$ myoblasts enhances their differentiation potential, implying that desmin is important for muscle differentiation in *Lmna*$^{-/-}$ myoblasts. This finding is particularly interesting given that desmin is a cytoplasmic intermediate filament protein not directly linked to myogenic transcription. However, vimentin and desmin intermediate filaments are known to stably associate with nuclear matrix attachment regions, where they might secondarily affect gene transcription (Tolstonog et al. 2002). Thus the reduced expression of desmin in *Lmna*$^{-/-}$ myoblasts could lead to compromised communication between the cytoplasm and the nuclear envelope, and this might be restored by overexpression of desmin in *Lmna*$^{-/-}$ cells. This raises the possibility that EDMD phenotypes in vivo might be offset by therapeutic approaches designed to elevate desmin levels in skeletal and cardiac muscle.

Lamin A/C, MyoD, and EDMD

Lmna$^{-/-}$ myoblasts and *MyoD*$^{-/-}$ primary myoblasts are remarkably similar (Sabourin et al. 1999), sharing many overlapping phenotypes, including delayed cell cycle withdrawal, reduced differentiation potential, and reduced desmin and M-cadherin. Unlike *Lmna*$^{-/-}$ mice, however, *MyoD*$^{-/-}$ mice do not develop a muscular dystrophy phenotype (Rudnicki et al. 1992). Why should populations of mutant myoblasts behave similarly in in vitro differentiation assays but differently in the organism? Interestingly, when *MyoD*$^{-/-}$ mice are challenged with acute muscle injury, they form few fully repaired fibers and the majority of the differentiated cells within the damaged area remain mononucleated. In addition, when *MyoD*$^{-/-}$ mice are interbred with mice that contain a naturally occurring loss-of-function mutation in the dystrophin gene (*mdx*) (Bulfield et al. 1984; Hoffman et al. 1987; Cox et al. 1993), they develop a more severe myopathy resulting in death at ~1 yr (Megeney et al. 1996). In contrast, *mdx* mice have a normal life span and exhibit little overt skeletal muscle weakness except in the diaphragm muscle. Thus, in mouse muscle disease models, dystrophic phenotypes are enhanced when increased degeneration (due to the absence of dystrophin or lamin A/C) is coupled with decreased regenerative potential due to the experimental removal of MyoD in *mdx/MyoD*$^{-/-}$ mice and the possible decrease of MyoD in *Lmna*$^{-/-}$ mouse satellite cells in vivo. A further complication is that Myf-5 protein levels are substantially increased in *Lmna*$^{-/-}$ myoblasts (Fig. 3A), and yet, the increased level of this highly related transcription factor does not compensate for the decreased level of MyoD.

Two models have been proposed to explain dystrophic syndromes of muscle resulting from *LMNA* mutation. One "structural weakness" model proposes that *LMNA* mutant myoblasts exhibit reduced cellular integrity and therefore enhanced tissue degeneration. Indeed, cells lacking lamin A/C exhibit impaired viability under mechanical strain as well as changes in expression of mechanosensitive genes (Broers et al. 2004; Lammerding et al. 2004). The "impaired differentiation" model proposes that tissue regeneration may be compromised in *Lmna*$^{-/-}$ muscle due to defective maintenance of the myogenic program in satellite cells. Our in vitro findings provide compelling evidence that muscle differentiation programs are compromised in *Lmna*$^{-/-}$ myoblasts, resulting in impaired myogenic potential. This conclusion is reinforced by the finding that MyoD protein levels are decreased in myoblasts deficient for lamin A/C and emerin, and that forced expression of MyoD in such cells restores muscle differentiation. We propose that both "structural weakness" and "impaired differentiation" defects contribute to muscular dystrophy in the murine *Lmna*$^{-/-}$ disease model.

Materials and methods

Derivation of Lmna$^{+/+}$, Lmna$^{+/-}$, *and* Lmna$^{-/-}$ *myoblast cell lines and conditions for differentiation*

Primary and permanent *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and *Lmna*$^{-/-}$ myoblasts were derived from 5-wk-old *Lmna*$^{+/+}$, *Lmna*$^{+/-}$, and

Lmna$^{-/-}$ mouse hindlimb muscles (provided by Colin Stewart, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). Hindlimb muscles were dissected in F10C medium (GIBCO) to isolate skeletal muscle and to remove adipose and connective tissues. Muscle was minced with fine scissors and digested with 375 U/mL type II collagenase (Worthington) for 1 h at 37°C and cultured as previously described (Hauschka et al. 1979; Neville et al. 1997). All cultures were grown on plates coated with 0.67% gelatin (Difco) in F10C medium supplemented with 15% preselected horse serum and fed 4 ng/mL basic fibroblast growth factor (human recombinant bFGF, Zymogenetics) every 12 h. Cells were passaged every 3 d and seeded at 5×10^4 cells per 100-mm plate continually until the spontaneously immortalized permanent line was generated. No individual muscle clones were isolated during derivation of the cell lines because we wanted the final cell populations to be maximally representative of all myogenic cells within each muscle sample. Cells in the 10–15 passage range following derivation of the permanent cell lines were used for all experiments.

Muscle cells were induced to differentiate via mitogen depletion, as described elsewhere (Clegg et al. 1987; Neville et al. 1997). Briefly, proliferating myoblasts are rinsed in saline G to remove residual bFGF before adding F10C supplemented with 1.5% horse serum and 1 μM insulin. For the clonal analysis of myogenesis (Clegg et al. 1987), *Lmna* muscle clones were switched to low mitogen media as above, but after 4 d, the cultures were switched back to F10C medium containing 15% horse serum and 1μM insulin for the duration of the experiment. This enhances long-term culture survival without stimulating cell replication, since bFGF is not present.

Differentiation and proliferation assays, immunohistochemistry, and Western blotting

To measure muscle differentiation, cells were fixed in alcohol–formalin–acetic acid (AFA); immunostained for MyHC using monoclonal MF-20 diluted 1:100 (gift from D.A. Fischman, Cornell University Medical College, New York), plus biotinylated rabbit anti-mouse IgG, streptavidin, and biotinylated horseradish peroxidase (Vector Labs, Inc.); and counterstained with 1% methylene blue or hematoxylin. For detecting S-phase cells, cultures were pulsed with BrdU (Amersham; 2 μL/mL of media) for 1 h prior to fixation as described elsewhere (Foster et al. 1987) and subsequent immunostaining using anti-BrdU diluted at 1:2000 (G3G4; gift from Steve Kaufman, University of Illinois, Urbana) and MF-20 diluted at 1:100 using isotype-specific secondary immunofluorescent antibodies (Molecular Probes). For high-resolution optical sectioning of myoblasts using the Zeiss axiovert 200M, cells were plated on 1- to 2-mm round glass coverslips (Fisher) that had been prepared by incubating 12–24 h in 1 M magnesium acetate, rinsed twice with PBS, then incubated for 1 h in 25 μg/mL poly-L-lysine (ICN Biomedicals), and finally incubated 3–24 h in 5 μg/mL laminin (Sigma). After plating, cells were fixed as described elsewhere (Kennedy et al. 2000; Barbie et al. 2004) and immunostained with the following antibodies: MyoD diluted at 1:500 (M-318; Santa Cruz), desmin diluted at 1:200 (clone D33; DAKO), and Pax7 diluted at 1:200 (developed by Atsushi Kawakami [University of Tokyo, Tokyo, Japan] and obtained from the Developmental Studies Hybridoma Bank, University of Iowa).

Muscle cells were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% deoxycholate) supplemented with phosphatase and protease inhibitors (1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 mM EDTA, 1 μg/mL aprotinin, 1 μg/mL leupeptin). Western analysis of muscle cell lysates was performed using standard procedures. The following antibodies were used: lamin A/C diluted at 1:1000 (2032; Cell Signaling), MyoD diluted at 1:100 (clone 5.8A; gift from Peter Houghton and Peter Diaz, St. Jude Children's Hospital, Memphis, TN), MyoD diluted at 1:100 (M-318; Santa Cruz), Myf-5 diluted at 1:100 (C-20; Santa Cruz), MEF2C diluted at 1:200 (9792; Cell Signaling), MEF2D diluted at 1:200 (clone 9; BD), pRB diluted at 1:100 (clone G3-245; BD), desmin diluted at 1:20 (clone D3; gift from D.A. Fischman, Cornell University Medical College), desmin diluted at 1:10,000 (ab15200; abcam), Pax3 diluted at 1:200 (gift from J.A. Epstein, Cardiovascular Division, University of Pennsylvania, Philadelphia, PA), Pax7 diluted at 1:50 (obtained from the Developmental Studies Hybridoma Bank, University of Iowa), β-catenin diluted at 1:1000 (H-102; Santa Cruz), emerin diluted at 1:1000 (FL-254; Santa Cruz), M-cadherin diluted at 1:300 (clone 5; BD), c-met diluted at 1:1000 (SP260; Santa Cruz), pan-actin diluted at 1:10,000 (clone C4; Chemicon), and β-actin diluted at 1:10,000 (clone ab8226; abcam).

Retroviral constructs and transduction

293T cells were acquired from ATCC and were cultured in DMEM supplemented with 10% fetal calf serum. RNAi constructs for mouse lamin A/C, mouse emerin, and enhanced green fluorescent protein (EGFP) were cloned into pSuper.retro-Puro as described elsewhere (Kudlow et al. 2005). Lamin A-SiR was generated by site-directed mutagenesis of a wild-type human lamin A cDNA using an oligonucleotide with the following sequence: GCAGACCATGAAGGAGGAGCTCGATTTTC AAAAGAATATCTACAGTGAGGAGCTGCG. The mutated lamin A cDNA was sequence-verified and subcloned into pMXIH for high-level, retroviral expression (Kudlow et al. 2005) as was wild-type lamin A. Both wild-type lamin A and lamin A-SiR were expressed as prelamin A (including the 18 amino acids that are eventually cleaved) so as to be processed naturally by the target cells. Desmin (IMAGE 4219280) was cloned into pMSCV-puro (Clontech) using BglII and EcoRI and was sequence-verified. pBABE- and pLXSH-MyoD-ER plasmids were gifts from Stephen J. Tapscott (Fred Hutchison Cancer Research Center, Seattle, WA). All retroviral constructs were transiently cotransfected into 293T cells with an ecotrophic packaging plasmid to generate nonreduplicating retroviruses. Viral supernatants from the 293T cells were filtered through 0.45-μm syringe filters (Millipore), added to exponentially growing *Lmna* muscle cultures (~200,000 cells per 100-mm plate), and supplemented with 4 ng/mL polybrene (Sigma). *Lmna* muscle cultures were retrovirally transduced either in F10C supplemented with 15% horse serum or at 1:1 ratios of 15% horse serum F10C and 10% fetal calf serum DMEM and fed 12 ng/mL bFGF for the duration of the retroviral infection. Selection for puromycin- or hygromycin-resistant myoblasts occurred after 24–36 h post-infection using 10 μg/mL and 300 μg/mL concentrations, respectively. Differentiation of MyoD-ER-*Lmna*$^{-/-}$ myoblasts and MyoD-ER-*Emd*RNAi *Lmna*$^{+/+}$ myoblasts was achieved by addition of 0.1 μM β-estradiol (Sigma) following a standard medium switch (see above) to differentiation media.

QPCR

mRNA's were purified using the RNeasy kit (Qiagen). Samples were DNase I-treated, and cDNA was generated using reverse transcriptase (Promega) and oligo dT15 primers (Invitrogen). For QPCR, 10 μL of 2× master mix containing SYBR Green (Applied Biosystems) was mixed with cDNA and 300 μM forward and reverse primers. Triplicates of the cDNA's were amplified on the Opticon I real-time thermal cycler (MJ Research). The experiments were performed three times at three different cDNA

dilutions. PCR products were normalized against the housekeeping gene *Hprt*, and measurements between samples were compared by cycle threshold (Ct).

Primer sequences used for QPCR are the following: *Hprt* (GenBank J00423) forward, 5′-AGGACCTCTCGAAGTGTT GG-3′; *Hprt* reverse, 5′-TGGCAACATCAACAGGACTC-3′; RB (GenBank NM_009029) forward, 5′-TACACTCTGTGCAC GCCTTC-3′; RB reverse, 5′-TCACCTTGCAGATGCCATAC -3′; MyoD (GenBank X61655) forward, 5′-CATCCGCTACATC GAAGGTC-3′; MyoD reverse, 5′-TAGTAGGCGGTGTCGTA GCC-3′; desmin (GenBank BC031760) forward, 5′-TACACCT GCGAGATTGATGC-3′; and desmin reverse, 5′-ACATCCAA GGCCATCTTCAC-3′.

Acknowledgments

We are grateful to Colin Stewart and Teresa Sullivan for providing *Lmna* mouse muscle tissue. We thank John Angello, Lindus Conlan, Erica Smith, Rob Welikson, and other members of the Hauschka and Kennedy Laboratories for helpful comments and suggestions. We also thank Joyce Hong, Bharti Sharma, and Nayan Arora for their help in maintaining and generating tissue culture reagents and performing MyHC staining, and Linnea Welton for technical assistance. B.A.K. is supported in part by PHS NRSA T32 GM07270 from NIGMS. This work was supported by the National Institutes of Health grant R01AG024287 to B.K.K., and by grants RO1AR18860 and 1PO1 NS046788 to S.D.H. B.K.K. is a Searle Scholar.

References

Agbulut, O., Li, Z., Perie, S., Ludosky, M.A., Paulin, D., Cartaud, J., and Butler-Browne, G. 2001. Lack of desmin results in abortive muscle regeneration and modifications in synaptic structure. *Cell Motil. Cytoskeleton* **49:** 51–66.

Anastasi, S., Giordano, S., Sthandier, O., Gambarotta, G., Maione, R., Comoglio, P., and Amati, P. 1997. A natural hepatocyte growth factor/scatter factor autocrine loop in myoblast cells and the effect of the constitutive Met kinase activation on myogenic differentiation. *J. Cell. Biol.* **137:** 1057–1068.

Angello, J.C. and Hauschka, S.D. 1996. Skeletal muscle satellite cells: Timelapse videomicroscopic evidence that renewal is stochastic. *BAM* **6:** 491–502.

Arimura, T., Helbling-Leclerc, A., Massart, C., Varnous, S., Niel, F., Lacene, E., Fromes, Y., Toussaint, M., Mura, A.M., Keller, D.I., et al. 2005. Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies. *Hum. Mol. Genet.* **14:** 155–169.

Bakay, M., Wang, Z., Melcon, G., Schiltz, L., Xuan, J., Zhao, P., Sartorelli, V., Seo, J., Pegoraro, E., Angelini, C., et al. 2006. Nuclear envelope dystrophics show a transcriptional fingerprint disruption of Rb–MyoD pathways in muscle regeneration. *Brain* (in press).

Barbie, D.A., Kudlow, B.A., Frock, R., Zhao, J., Johnson, B.R., Dyson, N., Harlow, E., and Kennedy, B.K. 2004. Nuclear reorganization of mammalian DNA synthesis prior to cell cycle exit. *Mol. Cell. Biol.* **24:** 595–607.

Bione, S., Maestrini, E., Rivella, S., Mancini, M., Regis, S., Romeo, G., and Toniolo, D. 1994. Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. *Nat. Genet.* **8:** 323–327.

Bonne, G., Di Barletta, M.R., Varnous, S., Becane, H.M., Hammouda, E.H., Merlini, L., Muntoni, F., Greenberg, C.R., Gary, F., Urtizberea, J.A., et al. 1999. Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. *Nat. Genet.* **21:** 285–288.

Broers, J.L., Peeters, E.A., Kuijpers, H.J., Endert, J., Bouten, C.V., Oomens, C.W., Baaijens, F.P., and Ramaekers, F.C. 2004. Decreased mechanical stiffness in *LMNA*$^{-/-}$ cells is caused by defective nucleo-cytoskeletal integrity: Implications for the development of laminopathies. *Hum. Mol. Genet.* **13:** 2567–2580.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550–553.

Bulfield, G., Siller, W.G., Wight, P.A., and Moore, K.J. 1984. X chromosome-linked muscular dystrophy (mdx) in the mouse. *Proc. Natl. Acad. Sci.* **81:** 1189–1192.

Buskin, J.N. and Hauschka, S.D. 1989. Identification of a myocyte nuclear factor that binds to the muscle-specific enhancer of the mouse muscle creatine kinase gene. *Mol. Cell. Biol.* **9:** 2627–2640.

Charge, S.B. and Rudnicki, M.A. 2004. Cellular and molecular regulation of muscle regeneration. *Physiol. Rev.* **84:** 209–238.

Clegg, C.H., Linkhart, T.A., Olwin, B.B., and Hauschka, S.D. 1987. Growth factor control of skeletal muscle differentiation: Commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. *J. Cell Biol.* **105:** 949–956.

Cornelison, D.D.W. 1998. Gene expression in wild-type and MyoD-null satellite cells: Regulation of activation, proliferation and myogenesis. Ph.D thesis, pp. 131–138, California Institute of Technology, Pasadena, CA.

Cornelison, D.D., Olwin, B.B., Rudnicki, M.A., and Wold, B.J. 2000. MyoD$^{-/-}$ satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Dev. Biol.* **224:** 122–137.

Cox, G.A., Cole, N.M., Matsumura, K., Phelps, S.F., Hauschka, S.D., Campbell, K.P., Faulkner, J.A., and Chamberlain, J.S. 1993. Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity. *Nature* **364:** 725–729.

Dreuillet, C., Tillit, J., Kress, M., and Ernoult-Lange, M. 2002. In vivo and in vitro interaction between human transcription factor MOK2 and nuclear lamin A/C. *Nucleic Acids Res.* **30:** 4634–4642.

Favreau, C., Higuet, D., Courvalin, J.C., and Buendia, B. 2004. Expression of a mutant lamin A that causes Emery-Dreifuss muscular dystrophy inhibits in vitro differentiation of C2C12 myoblasts. *Mol. Cell Biol.* **24:** 1481–1492.

Foster, R.F., Thompson, J.M., and Kaufman, S.J. 1987. A laminin substrate promotes myogenesis in rat skeletal muscle cultures: Analysis of replication and development using antidesmin and anti-BrdUrd monoclonal antibodies. *Dev. Biol.* **122:** 11–20.

Gao, J., Li, Z., and Paulin, D. 1998. A novel site, Mt, in the human desmin enhancer is necessary for maximal expression in skeletal muscle. *J. Biol. Chem.* **273:** 6402–6409.

Glass, C.A., Glass, J.R., Taniura, H., Hasel, K.W., Blevitt, J.M., and Gerace, L. 1993. The α-helical rod domain of human lamins A and C contains a chromatin binding site. *EMBO J.* **12:** 4413–4424.

Gruenbaum, Y., Margalit, A., Goldman, R.D., Shumaker, D.K., and Wilson, K.L. 2005. The nuclear lamina comes of age. *Nat. Rev. Mol. Cell. Biol.* **6:** 21–31.

Guo, C.S., Degnin, C., Fiddler, T.A., Stauffer, D., and Thayer, M.J. 2003. Regulation of MyoD activity and muscle cell dif-

ferentiation by MDM2, pRb, and Sp1. *J. Biol. Chem.* **278:** 22615–22622.

Hauschka, S.D., Linkhart, T.A., Clegg, C.H., and Merrill, C. 1979. Clonal studies of human and mouse muscle. In *In Muscle regeneration* (ed. A. Mauro), pp. 311–322. Raven Press, New York.

Hoffman, E.P., Brown Jr., R.H., and Kunkel, L.M. 1987. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. *Cell* **51:** 919–928.

Hollenberg, S.M., Cheng, P.F., and Weintraub, H. 1993. Use of a conditional MyoD transcription factor in studies of MyoD *trans*-activation and muscle determination. *Proc. Natl. Acad. Sci.* **90:** 8028–8032.

Huh, M.S., Parker, M.H., Scime, A., Parks, R., and Rudnicki, M.A. 2004. Rb is required for progression through myogenic differentiation but not maintenance of terminal differentiation. *J. Cell Biol.* **166:** 865–876.

Hutchison, C.J. and Worman, H.J. 2004. A-type lamins: Guardians of the soma? *Nat. Cell Biol.* **6:** 1062–1067.

Johnson, B.R., Nitta, R.T., Frock, R.L., Mounkes, L., Barbie, D.A., Stewart, C.L., Harlow, E., and Kennedy, B.K. 2004. A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation. *Proc. Natl. Acad. Sci.* **101:** 9677–9682.

Kang, J.S., Feinleib, J.L., Knox, S., Ketteringham, M.A., and Krauss, R.S. 2003. Promyogenic members of the Ig and cadherin families associate to positively regulate differentiation. *Proc. Natl. Acad. Sci.* **100:** 3989–3994.

Kaufman, S.J., George-Weinstein, M., and Foster, R.F. 1991. In vitro development of precursor cells in the myogenic lineage. *Dev. Biol.* **146:** 228–238.

Kaufmann, U., Kirsch, J., Irintchev, A., Wernig, A., and Starzinski-Powitz, A. 1999. The M-cadherin catenin complex interacts with microtubules in skeletal muscle cells: Implications for the fusion of myoblasts. *J. Cell. Sci.* **112:** 55–68.

Kennedy, B.K., Barbie, D.A., Classon, M., Dyson, N., and Harlow, E. 2000. Nuclear organization of DNA replication in primary mammalian cells. *Genes & Dev.* **14:** 2855–2868.

Kudlow, B.A., Jameson, S.A., and Kennedy, B.K. 2005. HIV protease inhibitors block adipocyte differentiation independently of lamin A/C. *Aids* **19:** 1565–1573.

Lammerding, J., Schulze, P.C., Takahashi, T., Kozlov, S., Sullivan, T., Kamm, R.D., Stewart, C.L., and Lee, R.T. 2004. Lamin A/C deficiency causes defective nuclear mechanics and mechanotransduction. *J. Clin. Invest.* **113:** 370–378.

Lassar, A.B., Buskin, J.N., Lockshon, D., Davis, R.L., Apone, S., Hauschka, S.D., and Weintraub, H. 1989. MyoD is a sequence-specific DNA binding protein requiring a region of myc homology to bind to the muscle creatine kinase enhancer. *Cell* **58:** 823–831.

Lazarides, E. and Hubbard, B.D. 1976. Immunological characterization of the subunit of the 100 A filaments from muscle cells. *Proc. Natl. Acad. Sci.* **73:** 4344–4348.

Li, H. and Capetanaki, Y. 1993. Regulation of the mouse desmin gene: Transactivated by MyoD, myogenin, MRF4 and Myf5. *Nucleic Acids Res.* **21:** 335–343.

Li, H., Choudhary, S.K., Milner, D.J., Munir, M.I., Kuisk, I.R., and Capetanaki, Y. 1994. Inhibition of desmin expression blocks myoblast fusion and interferes with the myogenic regulators MyoD and myogenin. *J. Cell Biol.* **124:** 827–841.

Li, Z., Colucci-Guyon, E., Pincon-Raymond, M., Mericskay, M., Pournin, S., Paulin, D., and Babinet, C. 1996. Cardiovascular lesions and skeletal myopathy in mice lacking desmin. *Dev. Biol.* **175:** 362–366.

Lim, R.W. and Hauschka, S.D. 1984. EGF responsiveness and receptor regulation in normal and differentiation-defective mouse myoblasts. *Dev. Biol.* **105:** 48–58.

Lloyd, D.J., Trembath, R.C., and Shackleton, S. 2002. A novel interaction between lamin A and SREBP1: Implications for partial lipodystrophy and other laminopathies. *Hum. Mol. Genet.* **11:** 769–777.

Maione, R., Fimia, G.M., Holman, P., Schaffhausen, B., and Amati, P. 1994. Retinoblastoma antioncogene is involved in the inhibition of myogenesis by polyomavirus large T antigen. *Cell Growth Differ.* **5:** 231–237.

Markiewicz, E., Dechat, T., Foisner, R., Quinlan, R.A., and C.J. Hutchison, C.J. 2002. Lamin A/C binding protein LAP2α is required for nuclear anchorage of retinoblastoma protein. *Mol. Biol. Cell* **13:** 4401–4413.

Markiewicz, E., Ledran, M., and C.J. Hutchison, C.J. 2005. Remodelling of the nuclear lamina and nucleoskeleton is required for skeletal muscle differentiation in vitro. *J. Cell. Sci.* **118:** 409–420.

McKinsey, T.A., Zhang, C.L., and Olson, E.N. 2002. MEF2: A calcium-dependent regulator of cell division, differentiation and death. *Trends Biochem. Sci.* **27:** 40–47.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., and Rudnicki, M.A. 1996. MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes & Dev.* **10:** 1173–1183.

Melcon, G., Kozlov, S., Cutler, D.A., Sullivan, T., Hernandez, L., Zhao, P., Mitchell, S., Nader, G., Bakay, M., Rottman, J.N., et al. 2006. Loss of emerin at the nuclear envelope disrupts the Rb1/E2F and MyoD pathways during muscle regeneration. *Hum. Mol. Genet.* (in press).

Mounkes, L.C. and Stewart, C.L. 2004. Aging and nuclear organization: Lamins and progeria. *Curr. Opin. Cell Biol.* **16:** 322–327.

Mounkes, L.C., Kozlov, S., Hernandez, L., Sullivan, T., and Stewart, C.L. 2003. A progeroid syndrome in mice is caused by defects in A-type lamins. *Nature* **423:** 298–301.

Neville, C., Rosenthal, N., McGrew, M., Bogdanova, N., and Hauschka, S. 1997. Skeletal muscle cultures. *Methods Cell Biol.* **52:** 85–116.

Nikolova, V., Leimena, C., McMahon, A.C., Tan, J.C., Chandar, S., Jogia, D., Kesteven, S.H., Michalicek, J., Otway, R., Verheyen, F., et al. 2004. Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C-deficient mice. *J. Clin. Invest.* **113:** 357–369.

Novitch, B.G., Mulligan, G.J., Jacks, T., and Lassar, A.B. 1996. Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in S and G2 phases of the cell cycle. *J. Cell Biol.* **135:** 441–456.

Novitch, B.G., Spicer, D.B., Kim, P.S., Cheung, W.L., and Lassar, A.B. 1999. pRb is required for MEF2-dependent gene expression as well as cell-cycle arrest during skeletal muscle differentiation. *Curr. Biol.* **9:** 449–459.

Olwin, B.B. and Hauschka, S.D. 1988. Cell surface fibroblast growth factor and epidermal growth factor receptors are permanently lost during skeletal muscle terminal differentiation in culture. *J. Cell Biol.* **107:** 761–769.

Oustanina, S., Hause, G., and Braun, T. 2004. Pax7 directs postnatal renewal and propagation of myogenic satellite cells but not their specification. *EMBO J.* **23:** 3430–3439.

Ozaki, T., Saijo, M., Murakami, K., Enomoto, H., Taya, Y., and Sakiyama, S. 1994. Complex formation between lamin A and the retinoblastoma gene product: Identification of the domain on lamin A required for its interaction. *Oncogene* **9:** 2649–2653.

Parker, M.H., Seale, P., and Rudnicki, M.A. 2003. Looking back to the embryo: Defining transcriptional networks in adult

myogenesis. *Nat. Rev. Genet.* **4:** 497–507.
Paulin, D. and Li, Z. 2004. Desmin: A major intermediate filament protein essential for the structural integrity and function of muscle. *Exp. Cell Res.* **301:** 1–7.
Petropoulos, H. and Skerjanc, I.S. 2002. β-Catenin is essential and sufficient for skeletal myogenesis in P19 cells. *J. Biol. Chem.* **277:** 15393–15399.
Puri, P.L., Iezzi, S., Stiegler, P., Chen, T.T.,. Schiltz, R.L., Muscat, G.E., Giordano, A., Kedes, L., Wang, J.Y., and Sartorelli, V. 2001. Class I histone deacetylases sequentially interact with MyoD and pRb during skeletal myogenesis. *Mol. Cell* **8:** 885–897.
Relaix, F., Rocancourt, D., Mansouri, A., and Buckingham, M. 2005. A Pax3/Pax7-dependent population of skeletal muscle progenitor cells. *Nature* **435:** 948–953.
Rober, R.A., Weber, K., and Osborn, M. 1989. Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: A developmental study. *Development* **105:** 365–378.
Rudnicki, M.A., Braun, T., Hinuma, S., and Jaenisch, R. 1992. Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* **71:** 383–390.
Sabourin, L.A., Girgis-Gabardo, A., Seale, P., Asakura, A., and Rudnicki, M.A. 1999. Reduced differentiation potential of primary MyoD$^{-/-}$ myogenic cells derived from adult skeletal muscle. *J. Cell Biol.* **144:** 631–643.
Smith, E.D., Kudlow, B.A., Frock, R.L., and Kennedy, B.K. 2005. A-type nuclear lamins, progerias and other degenerative disorders. *Mech. Ageing Dev.* **126:** 447–460.
Smythe, G.M., Davies, M.J., Paulin, D., and Grounds, M.D. 2001. Absence of desmin slightly prolongs myoblast proliferation and delays fusion in vivo in regenerating grafts of skeletal muscle. *Cell Tissue Res.* **304:** 287–294.
Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C.L., and Burke, B. 1999. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* **147:** 913–920.
Taniura, H., Glass, C., and Gerace, L. 1995. A chromatin binding site in the tail domain of nuclear lamins that interacts with core histones. *J. Cell Biol.* **131:** 33–44.
Tapscott, S.J., Davis, R.L., Thayer, M.J., Cheng, P.F., Weintraub, H., and Lassar, A.B. 1988. MyoD1: A nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts. *Science* **242:** 405–411.
Templeton, T.J. and Hauschka, S.D. 1992. FGF-mediated aspects of skeletal muscle growth and differentiation are controlled by a high affinity receptor, FGFR1. *Dev. Biol.* **154:** 169–181.
Tolstonog, G.V., Sabasch, M., and Traub, P. 2002. Cytoplasmic intermediate filaments are stably associated with nuclear matrices and potentially modulate their DNA-binding function. *DNA Cell Biol.* **21:** 213–239.
Weitzer, G., Milner, D.J., Kim, J.U., Bradley, A., and Capetanaki, Y. 1995. Cytoskeletal control of myogenesis: A desmin null mutation blocks the myogenic pathway during embryonic stem cell differentiation. *Dev. Biol.* **172:** 422–439.
Wright, W. 1984. Control of differentiation in heterokaryons and hybrids involving differentiation-defective myoblast variants. *J. Cell Biol.* **98:** 436–443.
Yablonka-Reuveni, Z., Rudnicki, M.A., Rivera, A.J., Primig, M., Anderson, J.E., and Natanson, P. 1999. The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Dev. Biol.* **210:** 440–455.
Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A., and Gallie, B.L. 1996. pRb controls proliferation, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes & Dev.* **10:** 3051–3064.
Zammit, P.S., Golding, J.P., Nagata, Y., Hudon, V., Partridge, T.A., and Beauchamp, J.R. 2004. Muscle satellite cells adopt divergent fates: A mechanism for self-renewal? *J. Cell Biol.* **166:** 347–357.
Zastrow, M.S., Vlcek, S., and Wilson, K.L. 2004. Proteins that bind A-type lamins: Integrating isolated clues. *J. Cell Sci.* **117:** 979–987.
Zeschnigk, M., Kozian, D., Kuch, C., Schmoll, M., and Starzinski-Powitz, A. 1995. Involvement of M-cadherin in terminal differentiation of skeletal muscle cells. *J. Cell Sci.* **108:** 2973–2981.