Emerin inhibits Lmo7 binding to the Pax3 and MyoD promoters and expression of myoblast proliferation genes

Zinaida Dedieī, Maureen Cetera, Tatiana V. Cohen and James M. Holaska

Introduction

The nuclear envelope is composed of two lipid bilayers, the outer nuclear membrane, which is contiguous with the endoplasmic reticulum, and the inner nuclear membrane. The inner nuclear membrane contains a variety of integral membrane proteins. Among the first inner nuclear membrane proteins identified were members of the LEM domain family of nuclear proteins, which are named for LAP2, emerin and MAN1 (Hetzer et al., 2005). The LEM domain mediates binding to the chromatin-associated protein barrier-to-autointegration factor (BAF) (Lee et al., 2001; Margalit et al., 2007; Shumaker et al., 2001). Emerin also binds directly to A-type lamins (Holaska et al., 2003; Lee et al., 2001). Lamins are nuclear intermediate filament proteins that form a nuclear-envelope-associated lattice, which is required for nuclear envelope integrity (Vlcek and Foisner, 2007). A-type lamins are also required for localization of emerin to the inner nuclear membrane (Hetzer et al., 2005).

Mutations in the gene encoding emerin cause X-linked Emery–Dreifuss muscular dystrophy (X-EDMD), which is characterized by progressive skeletal muscle wasting, life-threatening irregular heart rhythms and contractures of major tendons (Vlcek and Foisner, 2007). Muscle biopsies from both X-EDMD patients and mice lacking emerin show increased expression of muscle regeneration pathway components (Bakay et al., 2006; Melcon et al., 2006), suggesting that emerin regulates the differentiation of muscle stem cells, also called satellite cells, during muscle regeneration. Further supporting a role for emerin in myogenic differentiation, emerin-downregulated myoblasts exhibit significant differentiation defects (Frock et al., 2006). These findings support a model in which the skeletal muscle phenotype of EDMD is caused by the inability of satellite cells to differentiate properly and repair the damaged muscle.

Muscle regeneration is a multi-step process that repairs damaged muscle. Upon muscle injury, satellite cells are activated and begin proliferating. A small fraction of these cells will retain their original gene expression program and replenish the satellite cell niche. The remaining activated satellite cells, which express the transcription factors Pax3 and Myf5, will further differentiate into myoblasts that rapidly proliferate and express Pax3, Pax7, Myf5 and MyoD (Kuang and Rudnicki, 2008). Pax3 and Pax7 are satellite cell specification genes that help define the satellite cell niche (Kuang and Rudnicki, 2008) and directly regulate MyoD and Myf5 expression (Bajard et al., 2006; Brunelli et al., 2007; Maroto et al., 1997; Tajbakhsh et al., 1997). These proliferating myoblasts will then terminally differentiate and form myocytes, which express myogenin and other myocyte-specific genes. Myocytes do not express Pax3 or Myf5 (Kuang and Rudnicki, 2008). The committed myocytes will then fuse with the damaged myofiber and regenerate the muscle.

The MyoD and Pax3 promoters bind many regulatory proteins including basal transcription factors (Leibham et al., 1994; Pedraza-Alva et al., 1994; Tapscott et al., 1992), myogenic transcription...
factors (Kuang and Rudnicki, 2008; L’Honoré et al., 2007; Leibham et al., 1994; Pownall et al., 2002; Tapscott et al., 1992) and transcription repressors (Lee et al., 2006). There are three regulatory elements in the MyoD promoter: the proximal regulatory region (PRR; –275 to +20 nucleotides, relative to the transcription start site) (L’Honoré et al., 2007; Pownall et al., 2002; Tapscott et al., 1992), the distal regulatory region (DRR; –5.39 kb to –4.67 kb) (Tapscott et al., 1992) and the core enhancer (CER; –20 kb) (Goldhamer et al., 1992; Kucharczuk et al., 1999). The DRR and PRR primarily associate with transcription activators and the CER primarily associates with transcription repressors. The Pax3 promoter contains four known cis-regulatory elements, sites I, II, A and B. The POU transcription factors Oct1 and Brn2 bind sites I and II and activate Pax3 expression (Zhu and Pruitt, 2005). The TALE homeodomain transcription factors Pbx and Prep1 bind sites A and B and repress Pax3 expression (Zhu and Pruitt, 2005). However, the molecular mechanisms underlying the regulation of Pax3 expression by these transcription regulators during differentiation remain poorly understood.

We previously identified Lmo7 as an emerin-binding protein by affinity chromatography of HeLa nuclear lysates (Holaska et al., 2006). Genomic deletion of Lmo7 plus eight exons of an upstream gene, Uchl3, causes myopathic phenotypes in mice (Semenova et al., 2003). Lmo7 regulates the expression of many muscle genes (Holaska et al., 2006), including components of the proposed emerin-regulated muscle regeneration pathway (Bakay et al., 2006; Holaska et al., 2003). Lmo7 regulates the expression of many muscle genes (Holaska et al., 2006), including components of the proposed emerin-regulated muscle regeneration pathway (Bakay et al., 2006; Holaska et al., 2003). Lmo7A is the only isoform containing the predicted transactivation domain (TAD) (Fig. 1A). Because Lmo7A is the only isoform containing the predicted TAD and Lmo7A is expressed in skeletal muscle, we hypothesize that Lmo7A regulates muscle-specific gene expression. Supporting this hypothesis, Lmo7A expression during development is coincident with the two waves of skeletal muscle differentiation and development at embryonic day (e) 11.5 and e15.5 (Kurihara et al., 2002; Rozenblum et al., 2002; Semenova et al., 2003). Collectively, these observations suggest that Lmo7A regulates genes important for myogenic differentiation and muscle development.

Here, we analyzed C2C12 myoblast differentiation in Lmo7-downregulated myoblasts to test these hypotheses. C2C12 myoblasts were differentiated and stably transfected with Lmo7 with or without emerin (Fig. 1A). Diagram of the domain structure of Lmo7 splice variants. The Lmo7 C-terminal fragment used for binding studies is also shown. The epitopes for Lmo7 sera 4001, 5688 and 5690 used in these studies is also shown. NES, nuclear export sequence; NLS, nuclear localization sequence; TAD, transactivation domain; PDZ, PDZ domain; LIM, LIM domain. (B) C2C12 myoblast differentiation was induced by addition of DM, and the localization of Lmo7 and emerin were monitored by immunofluorescence microscopy using antibodies against Lmo7 (serum 4001) or emerin. d, days post differentiation. Scale bars: 10 μm. (C) Subcellular fractionation studies were performed on C2C12 myoblasts or differentiated myotubes. SDS-PAGE samples were run with whole cell lysates (L, Load), the nuclear fraction (N) and the cytosolic fraction (C), transfected onto nitrocellulose, and localization of Lmo7 was detected with Lmo7 antibodies (serum 5690). (D) Quadriceps muscles from wild-type mice were stained with Lmo7 antibodies (serum 5688) or dystrophin antibodies (Dys), which marks the plasma membrane. Lmo7 localizes to the nuclei of putative myogenic precursors (arrows) and the plasma membrane of mature muscle (arrowhead).
downregulated myoblasts also exhibited reduced expression of crucial myogenic differentiation markers, including Pax3, Pax7, Myf5 and MyoD. Interestingly, we found that Lmo7 binds to the promoters of Pax3, MyoD and Myf5, suggesting that Lmo7 directly regulates their expression. Lmo7 binding to and activation of these promoters was inhibited by emerin binding to Lmo7, providing a potential mechanism for how emerin inhibits Lmo7 activity.

Results

Lmo7 moves from the nucleus to the cytoplasm during myoblast differentiation

We have shown previously that Lmo7 localizes to the nucleus, cytoplasm and cell surface in HeLa cells and actively shuttles between these compartments (Holaska et al., 2006). Thus we monitored Lmo7 localization during C2C12 myoblast differentiation. C2C12 myoblasts were induced to differentiate by the addition of differentiation medium (DM) and Lmo7 localization was monitored every day for 8 days by confocal immunofluorescence microscopy. Antibodies against Lmo7 were used to determine whether Lmo7 and emerin colocalized at the nuclear envelope, as predicted from our previous work. Before differentiation Lmo7 localized primarily in the nucleoplasm of proliferating myoblasts, with a portion of Lmo7 localized at the nuclear envelope (Fig. 1B). By days 2 and 3 of differentiation a substantial portion of Lmo7 localized to the nuclear envelope, where it colocalized with emerin (Fig. 1B). Between day 4 and 6 of myogenic differentiation there was a shift in Lmo7 localization from the nucleus to the cytoplasm (Fig. 1B), which appeared to occur in a stepwise manner: at 4 days of differentiation the Lmo7 nucleoplasmic localization was lost and Lmo7 localized to the nuclear envelope and cytoplasm (Fig. 1B), a substantial amount of nuclear-envelope-associated Lmo7 was then lost by day five and Lmo7 localized exclusively to the cytoplasm by 6 days of differentiation (Fig. 1B).

We next wanted to determine whether Lmo7 localized similarly in skeletal muscle. We performed immunofluorescence microscopy on mouse quadriceps and found that Lmo7 localized predominantly to the nuclear envelope of mature fibers (Fig. 1D, arrowhead) and between the muscle fiber and the basal lamina (Fig. 1D, arrows). To confirm the relocalization of Lmo7 in differentiating C2C12 myoblasts we performed biochemical fractionation on C2C12 myoblasts and myotubes. In undifferentiated myoblasts, 96.4±5.7% of Lmo7 localized to the nucleus (Fig. 1C). In differentiated myotubes, 32.2±11.8% of Lmo7 was localized to the nucleus and 67.8±11.9% localized to the cytoplasm. A doublet was recognized by Lmo7 antibodies in the whole cell lysates (L) and the nuclear fractions (N), whereas only one band was seen in the cytoplasm (Fig. 1C). This suggests that the nucleus contains both post-translationally modified Lmo7 and unmodified Lmo7, whereas the cytoplasm contains only post-translationally modified Lmo7.

We next examined the expression of Lmo7 during myogenic differentiation. Expression of MyoD, myogenin and myosin heavy chain (MyHC) was also monitored to confirm proper myogenic differentiation. C2C12 myoblasts were incubated with DM for the indicated times and cells from each timepoint were suspended in sample buffer, separated by SDS-PAGE and subjected to western blotting with antibodies against the indicated proteins (Fig. 2A). Densitometry was then performed to quantify changes in protein expression. Lmo7 protein expression increased 1.6-fold after 1 day in DM and Lmo7 was upregulated 2.5-fold by two days after DM addition (Fig. 2A). Lmo7 remained at this level throughout the rest of differentiation. Emerin protein expression increased 1.3-fold after 2 days in DM, but returned to baseline by day 3.

To determine whether mRNA expression also showed a similar temporal expression pattern, we isolated RNA from C2C12 differentiating myoblasts everyday for 7 days and analyzed their expression by quantitative real-time PCR (qPCR). Expression of Lmo7, MyoD, myogenin and MyHC mRNA was normalized to that of GAPDH. Similar to Lmo7 protein expression, Lmo7 mRNA increased 2.6-fold after 1 day in DM (Fig. 2B). However, Lmo7 mRNA levels continued to increase during differentiation with highest expression at day 3 (Fig. 2B). MyoD, myogenin and MyHC all had increased mRNA expression during differentiation (Fig. 2C–E). The highest expression of MyoD mRNA and protein was seen after Lmo7 levels peaked, consistent with our hypothesis that Lmo7 activates MyoD expression. Importantly, the temporal activation of MyoD, myogenin and MyHC at both the protein and mRNA levels is consistent with proper C2C12 myogenic differentiation.

To confirm that only the largest muscle-specific Lmo7 isoform (Lmo7A) was expressed during C2C12 differentiation we reverse-transcribed RNA from differentiating C2C12 myoblasts and used the resulting cDNA for PCR using primers that spanned the region spliced out of Lmo7B and Lmo7C (Fig. 2F). Cloned Lmo7A plasmid DNA was used as a positive control. The majority product produced throughout differentiation was ~1100 bp (Fig. 2F), consistent with the presence of Lmo7A. A similar size band was seen in the positive control reaction. There was a faint band migrating at approximately 175 bp in samples from days 2–4 (Fig. 2F). The size of the predicted PCR product for Lmo7B or C is 101 bp. Thus we conclude that Lmo7A is the predominant Lmo7 isoform expressed throughout C2C12 differentiation.

The relocalization of Lmo7 to the nuclear periphery coincides with increased emerin expression (Fig. 1B and 2A). Thus we tested whether increased emerin expression was able to recruit Lmo7 to the nuclear envelope by expressing GFP or GFP–emerin in C2C12 myoblasts. Endogenous Lmo7 localized predominantly to the nucleoplasm in myoblasts expressing GFP (Fig. 2G), as expected. Expression of GFP–emerin caused a dramatic relocalization of endogenous Lmo7 from the nucleoplasm to the nuclear envelope (Fig. 2G). These results suggest that increased emerin expression between 2 and 4 days of myoblast differentiation causes the relocalization of Lmo7 to the nuclear envelope at this time.

Lmo7 is a transcription activator that regulates the expression of myoblast proliferation genes and myoblast differentiation

To test whether Lmo7 regulates myoblast differentiation, Lmo7 short hairpin RNA (shRNA) or control shRNA plasmids were expressed in C2C12 myoblasts, and differentiation was induced by the addition of DM. The shRNA plasmids express GFP from an internal ribosome entry site to mark the shRNA containing myoblasts and myotubes. Myotube formation was assessed by counting the number of GFP-positive myotubes that had >3 nuclei and expressed myosin heavy chain after 5 days in DM. A total of 50–75 GFP-positive cells were counted for each experiment (n=4); 53.0±12.9% of control myoblasts successfully formed myotubes after 5 days. Only 10.7±3.7% of Lmo7-downregulated myoblasts formed myotubes (Fig. 3A), demonstrating that Lmo7 is important for myotube formation. We
also noticed that at 3 days after addition of DM 82±4.9% of control myoblasts became elongated, which is a hallmark of early myogenic differentiation. Interestingly, only 20.3±9.0% of Lmo7-downregulated myoblasts exhibited an elongated phenotype, suggesting Lmo7 downregulation delays the early steps in myoblast differentiation, which results in defective myotube differentiation.

To test further whether Lmo7 downregulation caused differentiation delays, we examined the temporal expression of MyoD, myogenin and MyHC during differentiation of myoblasts expressing Lmo7 shRNA or control shRNA. Lmo7-downregulation in myoblasts caused a 64% decrease in Lmo7 expression. Lmo7 remained downregulated throughout differentiation (Fig. 3B), although Lmo7 expression increased after day 3 reaching a 33% decrease in Lmo7 expression by day 4. Importantly, proper activation and expression of MyoD, myogenin and MyHC were all delayed by 1 to 3 days (Fig. 3B). MyoD was reduced by 42% at 1 day of differentiation and did not reach wild-type expression levels until 4 days after differentiation. Myogenin and MyHC protein levels were reduced 83–89% at day 1 and 2, respectively. Myogenin and MyHC expression began to recover by day 4 (29% decreased), which is when Lmo7 expression begins to recover. Thus Lmo7 is required for proper temporal expression of myogenic factors important for differentiation.

Collectively, the above data suggested that Lmo7 regulates the expression of genes important for myoblast proliferation or early steps in myogenic differentiation, or both. To test this hypothesis, we measured mRNA levels of Pax3, Pax7, Myf5, MyoD and MyHC in C2C12 myoblasts expressing Lmo7 shRNA or control shRNA. RNA was isolated from Lmo7-downregulated or control myoblasts and measured by qPCR. Lmo7 downregulation resulted in a 60% decrease in Lmo7 mRNA expression. Importantly, Lmo7-downregulated myoblasts had substantially reduced expression of Myf5 (50%), MyoD (60%), Pax3 (53%) and Pax7 (55%; Fig. 3C, n=5). There was no substantial change in MyHC (Fig. 3C) or myogenin (data not shown) expression in Lmo7-downregulated myoblasts. To confirm that Lmo7 activates the expression of myogenic proliferation and early differentiation genes, we expressed GFP or GFP–Lmo7A in C2C12 myoblasts and measured the mRNA expression of Myf5 and MyoD by qPCR. GFP–Lmo7A expression increased expression of Myf5 6.2-fold and MyoD 4.1-fold (Fig. 3D), supporting our hypothesis that Lmo7 activates the expression of crucial myogenic differentiation genes. Thus, we conclude that the differentiation delay seen in Lmo7-downregulated myoblasts is caused by the failure to activate the expression of crucial myogenic genes.

Lmo7 binds the promoters of myoblast proliferation genes

We next tested whether Lmo7 binds the promoters of MyoD, Pax3 and Myf5. Chromatin immunoprecipitation (ChIP) assays were performed with anti-Lmo7 antibodies (serum 5688; J.M.H. and K. 1694 Journal of Cell Science 124 (10)
Lmo7 regulates myogenic differentiation

Wilson, unpublished results) or pre-immune serum (negative control) to test whether Lmo7 binds to the Pax3, Myf5 or MyoD promoters in C2C12 myoblasts (n=4). Multiple sets of primers within the promoter region of each of these genes were used to test whether Lmo7 bound their promoters (Fig. 4A). The myogenin promoter was used as a negative control. Anti-Lmo7 antibodies only efficiently pulled down the region of the Pax3 promoter from -1135 to -722 nucleotides, which contains two known regulatory elements (sites B and II), and failed to interact with promoter regions downstream of these sites (Fig. 4A). Lmo7 efficiently pulled down the region of the MyoD promoter from -478 to -288 nucleotides (Fig. 4A), which overlaps with the PRR, a positive regulatory element, but failed to pull-down the DRR and CER, two other well-characterized regulatory elements (Fig. 4A). Lmo7 also efficiently immunoprecipitated the region of the Myf5 promoter from -269 to -111 nucleotides, which overlaps with the promoter region of Myf5, but failed to pull-down the enhancer region (Fig. 4A). Two different primer sets that span the known regulatory elements of the myogenin promoter were used to test whether Lmo7 bound the myogenin promoter. Lmo7 failed to immunoprecipitate myogenin, as expected (Fig. 4A). Pre-immune serum failed to pull-down Pax3, Myf5, MyoD or myogenin promoters. We conclude that Lmo7 interacts with the Pax3, Myf5 and MyoD promoters and preferentially targets active regulatory elements.

We next tested whether Lmo7 bound directly to the MyoD or Pax3 promoters. Electrophoretic mobility-shift assays (EMSAs) were used to test whether Lmo7 bound to the Pax3 and MyoD promoters. Recombinant purified Lmo7 (5 μM) from the C-terminal region (Lmo7 C-term; residues 888–1683) or GST–Lmo7C1 (residues 1320–1412; negative control) was incubated with 200 ng MyoD promoter (nucleotides -478 to -288) in EMSA binding buffer. Non-denaturing PAGE was performed on each sample and DNA was detected with SYBR Green. MyoD promoter incubation with Lmo7 C-term substantially retarded MyoD promoter migration (Fig. 5A, n=5), demonstrating that the Lmo7 C-term binds directly to the MyoD promoter. GST–Lmo7C1 failed to bind the MyoD promoter, showing that the interaction between Lmo7 C-term and MyoD was specific. Lmo7 C-term (5 μM) was incubated with 200 ng myogenin promoter (nucleotides -289 to +7) as a negative control. Incubation of Lmo7 C-term with the myogenin promoter failed to retard myogenin promoter migration (Fig. 4B), further confirming that the interaction of Lmo7 C-term with MyoD was promoter-specific. The Pax3 promoter (200 ng, nucleotides -1135 to -722) was also incubated with 5 μM Lmo7 C-term or GST–Lmo7C1 in EMSA binding buffer to test whether Lmo7 C-term bound directly to Pax3. Lmo7 C-term efficiently bound the Pax3 promoter (Fig. 4C), whereas GST-Lmo7C1 failed to bind the Pax3 promoter.

Emerin binding inhibits Lmo7 binding to Pax3 and MyoD promoters
Emerin inhibits the activity of a number of transcription regulators, including Lmo7, β-catenin and GCL (Holaska et al., 2003; Holaska et al., 2006; Holaska and Wilson, 2006; Markiewicz et al., 2006). There are two models that can explain how emerin inhibits transcription regulator activity. The first is that emerin binds the transcription regulator at the nuclear envelope and inhibits its binding to DNA regulatory elements. The second model is that emerin binds to transcription regulators and recruits the regulators and the associated chromatin to the nuclear envelope. The repressive environment of the nuclear envelope would then be predicted to cause the associated chromatin to adopt a repressed structure. Because we had a transcription activator that bound emerin (Lmo7), and we had identified the promoter regions to which Lmo7 binds (in Pax3 and MyoD), we were able to directly test these models. The MyoD promoter (200 ng, -478 to -288) and Lmo7 C-term (1.5 μM) was incubated in the presence or absence of 4.2 μM wild-type emerin. These concentrations of emerin and Lmo7 C-term were used to ensure that >90% of Lmo7 C-term will be emerin-bound. Incubation of Lmo7 C-term with wild-type emerin inhibited Lmo7 C-term binding to the MyoD promoter (Fig. 5A, n=7). To test whether emerin binding to Lmo7 C-term was required for inhibiting Lmo7 C-term binding to the MyoD promoter, we incubated an emerin mutant that fails to bind Lmo7 C-term (emerin m179) (Holaska et al., 2006) with Lmo7 C-
term and the MyoD promoter. Incubation of Lmo7 C-term with emerin m179 failed to inhibit Lmo7 C-term binding to the MyoD promoter (Fig. 5A), demonstrating that emerin binding to Lmo7 C-term competes with Lmo7 C-term binding to the MyoD promoter. Importantly, neither wild-type emerin nor emerin m179 interacted with the MyoD promoter (Fig. 5A). We next tested whether wild-type emerin or emerin m179 inhibits Lmo7 C-term binding to the Pax3 promoter (nucleotides –1135 to –722). Addition of 1.7 µM or 4.2 µM wild-type emerin with 1.5 µM Lmo7 C-term and 200 ng Pax3 promoter blocked Lmo7 C-term binding to the Pax3 promoter (Fig. 5B, n=3). Incubation of 1.5 µM Lmo7 C-term with 1.7 µM or 4.2 µM emerin mutant m179 had no effect on Lmo7 C-term binding to the Pax3 promoter (Fig. 5B). Neither wild-type emerin nor emerin mutant m179 bound to the Pax3 promoter.

We hypothesized that expression of wild-type emerin would inhibit MyoD and Myf5 expression because emerin inhibits Lmo7 promoter binding. Furthermore, we predicted that expression of an emerin mutant that fails to bind Lmo7 would have no effect on MyoD or Myf5 expression. Hence, GFP–emerin or a GFP–emerin disease-causing mutant (P183H), which fails to bind Lmo7 (Holaska et al., 2006), was expressed in C2C12 myoblasts and the levels of Myf5 and MyoD mRNA were measured by qPCR. Expression of GFP–emerin decreased Myf5 and MyoD mRNA expression 1.8-fold (Fig. 5C, n=4). Importantly, expression of emerin P183H had no effect on Myf5 and MyoD expression, demonstrating that emerin binding to Lmo7 is necessary for inhibition of Lmo7 activity. Both GFP–emerin and GFP–emerin P183H were expressed at similar levels: 12.28±4.0- and 13.85±0.95-fold over endogenous emerin levels, respectively. Thus emerin binding to Lmo7 inhibits the binding of Lmo7 to myoblast proliferation promoters in vitro and the expression of myoblast proliferation genes in vivo.

We next examined the expression of MyoD in C2C12 myoblasts downregulated for Lmo7, emerin, or both emerin and Lmo7, to confirm that emerin inhibits Lmo7 activity. Downregulation of Lmo7 decreased MyoD expression 42% (Fig. 5D), as expected. Emerin downregulation increased expression of MyoD 1.5-fold (Fig. 5D), consistent with emerin inhibiting MyoD expression. C2C12 myoblasts downregulated for both Lmo7 and emerin rescued the expression of MyoD (Fig. 5D). Increased MyoD expression was calculated in both emerin-downregulated and Lmo7-downregulated backgrounds and in both cases MyoD expression was at normal myoblast levels (Fig. 5D). To confirm these results we performed gene expression profiling on primary emerin-null mouse myoblasts. Expression of Pax7, Pax3 and Myf5 was increased 2.9-fold, 1.7-fold and 2.9-fold, respectively. We next
tested whether expression of wild-type emerin, but not the emerin mutant that cannot bind Lmo7, rescued expression of Pax3, Pax7 and Myf5 in the emerin-null myoblasts. We expressed GFP, GFP–emerin or GFP–emerin P183H in emerin-null myoblasts and measured expression of MyoD, Myf5, Pax3 and Pax7. The transfection efficiency of each construct was ~30–40% (data not shown). Similar to the findings upon overexpression of these constructs in C2C12 myoblasts, expression of GFP–emerin caused a 50–66% decrease in expression of MyoD, Myf5, Pax3 and Pax7 (Fig. 5E), which restored their expression to wild-type levels. Expression of GFP–emerin P183H failed to downregulate expression of MyoD, Myf5, Pax3 or Pax7 (Fig. 5E).

Discussion
The results presented here show that Lmo7 regulates C2C12 myoblast differentiation by activating the expression of crucial myoblast proliferation and differentiation genes. Lmo7 binds directly to the Pax3 and MyoD promoters, suggesting that Lmo7 regulates the expression of these genes in myoblasts by binding directly to their promoters and activating their expression. Interestingly, emerin binding to Lmo7 inhibits Lmo7 binding to, and expression of, the Pax3 and MyoD promoters. This is the first study to demonstrate that emerin binding to a transcription regulator inhibits the interaction of transcription regulators with their promoters. Collectively, these studies indicate that emerin regulates myogenic differentiation by inhibiting Lmo7 binding to, and activation of, crucial myogenic differentiation genes (Fig. 6). We propose that improper activation of Lmo7 caused by the loss or mutation of emerin contributes to the defective muscle regeneration seen in X-EDMD.

Regulation of myogenic differentiation
It has become increasingly clear that Pax3 and Pax7 are critical components for regulating satellite cell proliferation and
**Fig. 6. Model for Lmo7 regulation of myogenic differentiation.** In myoblasts Lmo7 is predominantly nucleoplasmic, where it is free to bind the promoters of Pax3, Pax7, MyoD and Myf5 and activate their expression. Early in differentiation (0–2 days) Lmo7 expression increases and remains primarily nucleoplasmic. We propose that Lmo7 binds myogenic promoters early in differentiation and activates the expression of MyoD and other early differentiation genes, given that Lmo7 is predominantly nucleoplasmic. As differentiation proceeds, we propose that the affinity of emerin for Lmo7 increases and competes with Lmo7 binding to myogenic promoters. Localization of Lmo7 to the nuclear periphery and decreased expression of MyoD and Myf5 that occurs later in differentiation are both compatible with this model. Lmo7 is predominantly cytoplasmic in myotubes and we predict that there is a change in the nuclear transport dynamics that is at least partially responsible for this relocalization. This may also be coupled to increased affinity or expression of a cytoplasmic Lmo7-binding partner.

**Regulation of the Pax3 and MyoD promoters**

How do we envision Lmo7 activating MyoD expression during differentiation? On the basis of our data we predict that Lmo7 localization to the nuclear envelope during differentiation, through binding to emerin, inhibits Lmo7 binding to the MyoD promoter and activation of MyoD expression. This is supported by our finding that emerin competes with Lmo7 binding to the MyoD promoter in vitro. Furthermore, MyoD expression begins to decrease between 4 and 6 days of myogenic differentiation (Rudnicki et al., 2008), which is concomitant with Lmo7 localization to the nuclear envelope and cytoplasm. This model also predicts that, upon satellite cell activation, the concentration of free nucleoplasmic Lmo7 would increase dramatically to activate Myf5, Pax3, and MyoD expression. Alternatively, there might be an increase in the expression or localization of an undefined Lmo7 transcriptional co-activator upon satellite cell activation. If the first model is correct, we predict that Lmo7 is either cytoplasmic or nuclear-envelope-localized (emerin-bound) in quiescent satellite cells and Lmo7 would move to the nucleoplasm upon satellite cell activation. Future studies will be needed to examine the expression and localization of Lmo7 in quiescent and activated satellite cells in vivo, as these studies were performed in proliferating myoblasts.

In this study, we have shown that Lmo7 binds a region of the MyoD promoter that is juxtaposed to the PRR, which binds many transcription factors including AP-1, TBP and MEF2 (Leibham et al., 1994; Pedraza-Alva et al., 1994; Tapscott et al., 1992). The Lmo7-binding region of the MyoD promoter contains three sequences that are similar to the consensus E-box sequence. This region also contains putative binding sites for over 20 transcription factors, including AP-1, PPARα, IRF1 and IRF2. It will be interesting to determine whether Lmo7 binding to this region of the MyoD promoter competes with these transcription factors or whether they cooperatively bind to the promoter.

The Pax3 promoter region that binds Lmo7 contains two known regulatory regions: site II and B. However, alignment of the MyoD and Pax3 promoter elements predicts Lmo7 binds site II of the Pax3 promoter (J.M.H., unpublished). Thus, we hypothesize that...
Lmo7 competes with Oct1 or Brn2 for binding to and activation of the Pax3 promoter during myogenic differentiation. Oct1 and Brn2 are POU transcription factors that bind this region of the Pax3 promoter and activate Pax3 expression (Zhu and Pruitt, 2005). Alternatively, Lmo7 might cooperatively bind with Oct1 and Brn2 on the Pax3 promoter to activate Pax3 expression. Determining how Lmo7 binding to, and the subsequent activation of, the Pax3 and MyoD promoters is regulated will be vital for understanding how Lmo7 regulates myogenic progenitor cell activation, proliferation, differentiation and muscle regeneration.

Regulation of Lmo7 nuclear localization during myogenic differentiation

Here, we demonstrate that Lmo7 moves from the nucleus to the cytoplasm upon myoblast differentiation. We have previously shown that Lmo7 actively shuttles between the nucleus and cytoplasm (Holaska et al., 2006). Thus we predict that increased cytoplasmic Lmo7 during differentiation results, at least in part, from changes in nuclear transport dynamics. Interestingly, increased Lmo7 cytoplasmic localization coincides with reduced expression of myogenic progenitor proliferation genes (Rudnicki et al., 2008) and we hypothesize that Lmo7 nuclear transport is regulated during differentiation to regulate access of Lmo7 to promoters. Lmo7 cytoplasmic localization coincides with reduced expression of genes within this region of chromatin. As determined by western blotting (J.M.H., unpublished). Given that emerin is exclusively nuclear-envelope-associated, this creates a local concentration of 5.6 mM. The affinity (Kd) of Lmo7 C-term for emerin is 125 nM (Holaska et al., 2006). On the basis of the concentrations of Lmo7 and emerin in the nucleus, >99% of Lmo7 predicted to be emerin-bound. However, only 10% of Lmo7 localizes to the nuclear envelope in myoblasts, suggesting that Lmo7 binding to emerin is regulated by post-translational modification or emerin occupancy by other binding partners [e.g. β-catenin, GCL, Btf and the nuclear hormone receptor corepressor (NCoR) complex]. Lmo7 localization at the nuclear envelope also increased during differentiation. We predict that post-translational modification of Lmo7 or emerin, or both, increases their affinity for one another. Emerin contains four serine, one threonine and 13 tyrosine residues that are phosphorylated in vitro and in vivo (Amanchy et al., 2005; Brill et al., 2004; Hirano et al., 2005; Roberts et al., 2006; Schlosser et al., 2006), many of which regulate binding to its partners (Hirano et al., 2005; Roberts et al., 2006; Tiffert et al., 2009). However, it is not well documented how emerin phosphorylation changes during myogenic differentiation. Lmo7 is also phosphorylated on multiple residues (Beausoleil et al., 2006; Dephoure et al., 2008). It will be interesting to analyze Lmo7 and emerin phosphorylation during myogenic differentiation and determine how these post-translational modifications affect their interaction. Of course, it cannot be ruled out that the localization of Lmo7 is regulated by binding to other nuclear envelope proteins.

There are multiple proposed mechanisms for how nuclear envelope proteins regulate gene expression. MAN1, a LEM domain inner nuclear envelope protein, antagonizes the TGF-β pathway (Lin et al., 2005; Osada et al., 2003; Pan et al., 2005; Raju et al., 2003). It was initially proposed that MAN1 inhibits the TGF-β pathway by altering the phosphorylation status of the SMAD2–SMAD3 heterodimer (Pan et al., 2005), but this hypothesis remains to be rigorously tested. Nuclear envelope proteins have also been shown to regulate the nuclear localization of transcription factors. Hutchison and colleagues have demonstrated that loss of emerin increases nuclear β-catenin localization, resulting in increased β-catenin-dependent transcription (Markiewicz et al., 2006) and defective adipogenic differentiation (Tilgner et al., 2009). Another hypothesis is that emerin binding to transcription factors localizes the transcription-factor-bound chromatin to the repressed environment of the nuclear envelope. This localization is predicted to cause the chromatin to adopt a repressed structure, resulting in reduced expression of genes within this region of chromatin.
Although there is no direct evidence supporting this model, emerin and LAP2β were found to interact with histone deacetylase complexes (Holaska and Wilson, 2007; Somech et al., 2005). Furthermore, chromatin in cells lacking emerin adopts a more open conformation, (Boyle et al., 2001; Fidzianska and Hausmanowa-Petrusewicz, 2003). The final model, which the results presented here support, is that emerin binds to transcription factors (e.g. Lmo7) at the nuclear envelope and competes with transcription factor binding to regulatory elements (e.g. the MyoD promoter). We propose that emerin utilizes all of these mechanisms to regulate transcription factor activity, although it remains to be determined whether the mechanisms used by emerin to inhibit gene expression are pathway-dependent.

This is the first study to provide a mechanism for how loss of emerin might result in increased MyoD expression (i.e. by failing to inhibit Lmo7 activity). Supporting the competition model for emerin inhibition of Lmo7 activity, we found that emerin binding to Lmo7 inhibits Lmo7 binding to the MyoD and Pax3 promoters, resulting in decreased expression of MyoD and Pax3. We predict that emerin binding to Lmo7 will also inhibit binding to, and activation of, the Pax7 and Myf5 promoters, in the same manner as expression of exogenous emerin reduces Myf5 expression in myoblasts. On the basis of the results presented here, we hypothesize that Lmo7 regulation of Pax3, Pax7, and Myf5 expression is important to ensure proper myogenic differentiation during development and regeneration of damaged muscle. We further predict that loss of emerin expression or function, as is seen in X-EDMD patients, results in sustained activation of myogenic progenitor proliferation genes by failing to inhibit Lmo7 activity.

Failure to inhibit Lmo7 activity would then be predicted to result in the failure of myogenic progenitors to temporarily regulate the expression of crucial myogenic differentiation genes, including Pax3. In support of this model, aberrant expression of Pax3 in satellite cells delays myogenic differentiation (Boutet et al., 2007). This model predicts that emerin-null myoblasts will exhibit greater Lmo7 binding to other satellite cell activation and myoblast proliferation promoters, enhanced myogenic progenitor cell proliferation and exhaustion of the satellite cell niche by increased proliferation of myogenic progenitors that fail to fuse (Boutet et al., 2007). Future experiments will be needed to test whether satellite cells or myogenic precursors derived from X-EDMD patients and emerin-null mice have increased Pax3, Myf5 or Pax3 expression and exhaustion of the satellite cell niche, which would be predicted by these studies.

Materials and Methods

Expression and localization of myogenic proteins during myoblast differentiation

C2C12 myoblasts were seeded onto coverslips and maintained in growth medium [Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS)]. To induce differentiation, C2C12 myoblasts were incubated with differentiation medium (DM; DMEM + 2% horse serum) for 0–7 days. Cells were recovered every day and resuspended in SDS-PAGE sample buffer at a concentration of 106 nuclei per ml. A total of 10,000–50,000 nuclear equivalents were separated recovered every day and resuspended in SDS-PAGE sample buffer at a concentration of 106 nuclei per ml. A total of 10,000–50,000 nuclear equivalents were separated recovered every day and resuspended in SDS-PAGE sample buffer at a concentration of 106 nuclei per ml. A total of 10,000–50,000 nuclear equivalents were separated recovered every day and resuspended in SDS-PAGE sample buffer at a concentration of 106 nuclei per ml. A total of 10,000–50,000 nuclear equivalents were separated.

Promoter regions of MyoD (nucleotides –478 to –288), Pax3, Myf5 and myogenin promoters were myogenin promoter fragments were then purified using the Qiagen Gel Extraction Kit (Qiagen). 5 μM His6-tagged Lmo7 C-term (residues 888–1683) or 5 μM GST-tagged Lmo7C1 (residues 1320–1412) was incubated with 200 ng of each promoter fragment in EMSA binding buffer (10 mM Tris-Cl pH 7.4, 150 mM KCl, 0.1 mM EDTA, 0.5 mM DTT) for 5 minutes and subjected to electrophoresis. For experiments including emerin (residues 1–222), 1.5 μM His-Lmo7-C term and 4.2 μM emerin (residues 1–222) were incubated together with 200 ng of MyoD or Pax3 promoter fragment for 30 minutes. The recombinant emerin protein used in these assays contains the entire nucleoplasmic domain of emerin, which is sufficient to bind Lmo7 with high affinity (Holaska et al., 2006). Recombinant emerin and Lmo7C1-vena were purified and extensively described (Holaska et al., 2002; Holaska et al., 2006). DNA was visualized by staining with SYBR Green and protein was visualized using SYPRO-RUBY according to the manufacturer’s instructions (Invitrogen).

Chromatin immunoprecipitation

Chip assays were performed according to the manufacturer’s instructions (Millipore) using 106 C2C12 myoblasts per reaction and shearing the DNA to 200–750 bp by sonication with 10 pulses of 20 seconds each on setting 3 (Fisher 350 sonic dismembrator). A total of 2 μl of serum 5688 against Lmo7 or pre-immune serum was used for each reaction. Pax3, Myf5, MyoD and myogenin promoters were detected by amplification of input and immunoprecipitated DNA using primers specific to Pax3, Myf5, MyoD and myogenin regulatory elements (supplementary material Table S1).

Electrophoretic mobility-shift assays

EMSA were performed according to the manufacturer’s instructions (Invitrogen). Promoter regions of MyoD (nucleotides –478 to –288), Pax3 (nucleotides –1135 to –722) and the myogenin promoter (nucleotides –289 to +7) were generated by PCR using the primers listed in supplementary material Table S1. MyoD, Pax3 and myogenin promoter fragments were then purified using the Qiagen Gel Extraction Kit (Qiagen). 5 μM His6-tagged Lmo7 C-term (residues 888–1683) or 5 μM GST-tagged Lmo7C1 (residues 1320–1412) was incubated with 200 ng of each promoter fragment in EMSA binding buffer (10 mM Tris-Cl pH 7.4, 150 mM KCl, 0.1 mM EDTA, 0.5 mM DTT) for 5 minutes and subjected to electrophoresis. For experiments including emerin (residues 1–222), 1.5 μM His-Lmo7-C term and 4.2 μM emerin (residues 1–222) were incubated together with 200 ng of MyoD or Pax3 promoter fragment for 30 minutes. The recombinant emerin protein used in these assays contains the entire nucleoplasmic domain of emerin, which is sufficient to bind Lmo7 with high affinity (Holaska et al., 2006). Recombinant emerin and Lmo7C1-vena were purified and extensively described (Holaska et al., 2002; Holaska et al., 2006). DNA was visualized by staining with SYBR Green and protein was visualized using SYPRO-RUBY according to the manufacturer’s instructions (Invitrogen).
We thank Katherine Wilson (Johns Hopkins Medical School) for the generous gift of sera 5688 and 5690 against Lmo7. We thank Megan Roy-Puckelwartz and Yuan Zhang for their assistance with immunofluorescence microscopy of mouse skeletal muscle. We thank Elizabeth McNally, Megan Roy-Puckelwartz, Harinder Singh, Robert Goldman and members of the Holaska laboratory for many fruitful discussions. The American Heart Association (0830033N to J.M.H.), Goldman and members of the Holaska laboratory for many fruitful discussions. The American Heart Association (0830033N to J.M.H.), the Ellison Medical Foundation (J.M.H.) and the Schweppe Foundation (J.M.H.) supported this work.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/10/1691/DC1

References


Table S1. List of primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Set</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lmo7</td>
<td>isoform PCR</td>
<td><code>aagaggccagacaaacagaggt</code></td>
<td><code>atccgttggtacacgacggctc</code></td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td><code>ttctgaacatctcttgcgtgaca</code></td>
<td><code>tcgccccaaatcggagtgtgctctc</code></td>
</tr>
<tr>
<td>Emerin</td>
<td>qPCR</td>
<td><code>accccttctcttcccccttc</code></td>
<td><code>aagacagacaaaggcagcacaagacc</code></td>
</tr>
<tr>
<td>Pax3</td>
<td>ChIP set 1</td>
<td><code>ctgatctctcaaacctttgaag</code></td>
<td><code>gtgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 2</td>
<td><code>ctcatcctttgtgaattgattaagg</code></td>
<td><code>ctgcccatcctctttgatagatctgctggt</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 3</td>
<td><code>tgcccctctgttttgatagatctgctaaa</code></td>
<td><code>aagagacagacacaaaggcagcacaagacc</code></td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td><code>atctgtgcacagcgaggccttc</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>EMSA</td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
<td></td>
</tr>
<tr>
<td>MyoD</td>
<td>ChIP set 1</td>
<td><code>ccctggctcaacactttctg</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 2</td>
<td><code>tcagggacagcagactgtcct</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 3</td>
<td><code>ggccatctccgtatttagtactccggacaagc</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EMSA</td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
<td></td>
</tr>
<tr>
<td>Myf5</td>
<td>ChIP set 1</td>
<td><code>aagtctttctccagctgctg</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 2</td>
<td><code>ttggctctcttccccgtgct</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 3</td>
<td><code>agcctgtgtgctggatgtagctgct</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
<td></td>
</tr>
<tr>
<td></td>
<td>EMSA</td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
<td></td>
</tr>
<tr>
<td>Pax7</td>
<td>qPCR</td>
<td><code>ttgagggagggaggtggtggactggtctg</code></td>
<td><code>tgaggaggaggtggtggactggtctgga</code></td>
</tr>
<tr>
<td>Myogenin</td>
<td>ChIP set 1</td>
<td><code>gaacctgatcatcactccactg</code></td>
<td><code>accccaactgctggtggtgcca</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 2</td>
<td><code>aatcactctgatcactccactgg</code></td>
<td><code>ctctcctgtgatcatgac</code></td>
</tr>
<tr>
<td></td>
<td>ChIP set 3</td>
<td><code>ggccagactctgactttgatgctgt</code></td>
<td><code>tcagggacagctttaccacacca</code></td>
</tr>
<tr>
<td></td>
<td>qPCR</td>
<td><code>accccaactgctggtggtgcca</code></td>
<td><code>accccaactgctggtggtgcca</code></td>
</tr>
<tr>
<td></td>
<td>EMSA</td>
<td><code>accccaactgctggtggtgcca</code></td>
<td><code>accccaactgctggtggtgcca</code></td>
</tr>
<tr>
<td>MyHC</td>
<td>qPCR</td>
<td><code>acggccatcaggtacaagagaaga</code></td>
<td><code>tgagggtcttggaggtggtggttt</code></td>
</tr>
<tr>
<td>GAPDH</td>
<td>qPCR</td>
<td><code>aacctgggtggtggtggtggttt</code></td>
<td><code>tgagggtcttggaggtggtggttt</code></td>
</tr>
</tbody>
</table>