

Chapter 11

Analysis of Nuclear Lamina Proteins in Myoblast Differentiation by Functional Complementation

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Abstract

We describe straightforward methodology for structure-function mapping of nuclear lamina proteins in myoblast differentiation, using populations of C2C12 myoblasts in which the endogenous lamina components are replaced with ectopically expressed mutant versions of the proteins. The procedure involves bulk isolation of C2C12 cell populations expressing the ectopic proteins by lentiviral transduction, followed by depletion of the endogenous proteins using siRNA, and incubation of cells under myoblast differentiation conditions. Similar methodology may be applied to mouse embryo fibroblasts or to other cell types as well, for the identification and characterization of sequences of lamina proteins involved in functions that can be measured biochemically or cytologically.

Key words Nuclear lamina, Lentivirus, siRNA, Myoblast, Functional complementation

1 Introduction

The nuclear lamina, a filamentous protein meshwork that lines the nucleoplasmic surface of the nuclear envelope (NE), comprises a polymeric array of nuclear lamins and associated transmembrane and peripheral proteins (*reviewed in refs. 1–3*). The lamina has been a topic of rapidly expanding interest over the last 20 years. In significant part, this is due to the discovery that over 15 human diseases are linked to mutations in nuclear lamins and lamina-associated proteins (which collectively may be termed “laminopathies” or “nuclear envelopathies”) (*reviewed in refs. 4–6*). Whereas the majority of these diseases are caused by mutations in the gene encoding lamins A/C, similar pathologies can be caused by mutations in lamina-associated transmembrane proteins such as emerin [4–6]. This suggests an intimate functional connection between these physically linked components. Most disease-causing mutations in nuclear lamina proteins affect striated muscle, but they also can target adipose tissue, bone, neurons, or multiple organ systems such as seen with Hutchinson-Gilford progeria syndrome [4–6].

One of the major challenges in the field is to understand the molecular basis for disease caused by lamina protein mutations. Achieving this goal likely will require a detailed structure-function analysis of individual nuclear lamina proteins.

Since mutations in lamina components most commonly affect striated muscle, we have screened lamina-associated proteins for a potential role in muscle biology using cultured myoblast differentiation assays. Our work identified three new NE transmembrane proteins involved in myoblast differentiation: Lem2 [7], Net37 [8], and Net39 [9]. This work extended studies revealing that lamins A/C and emerin have a role in cultured myoblast differentiation [7, 10] and suggests that a diverse range of nuclear lamina proteins have important functions in the muscle. We have used the well-studied C2C12 murine myoblast cell line for these functional studies, as C2C12 cells readily differentiate into myotubes in culture (Fig. 1) and have been shown to closely recapitulate the myogenic differentiation program observed with primary myoblast cultures [11, 12].

In developing methods for ectopic expression of nuclear lamina proteins in C2C12 cells, we found that lentiviral methods are superior in many respects to conventional lipid-based transient transfection approaches [13]. In our hands, ectopic protein expression by lipid transfection yields highly variable cell-to-cell expression levels, and the ectopic proteins often accumulate in large cytoplasmic aggregates as well as at the NE in the majority of cells, regardless of the expression level (shown in Fig. 2a for Lem2). By contrast,

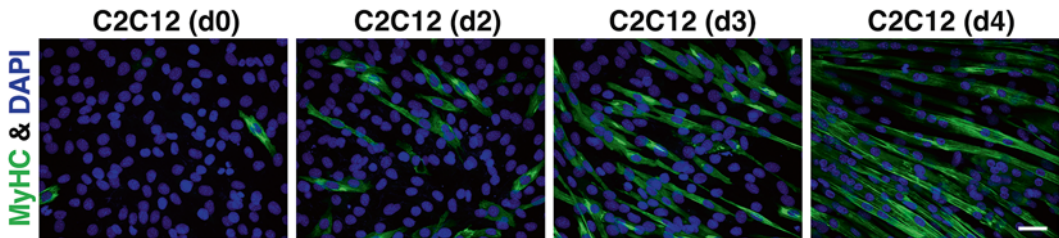


Fig. 1 The C2C12 myoblast differentiation model. Micrographs of C2C12 cells showing immunofluorescence (IF) labeling of skeletal myosin heavy chain (MyHC), a typical marker of terminally differentiated myotubes (*green*, MyHC; *blue*, DNA). MyHC is virtually absent in C2C12 cultures grown to confluency before differentiation (d0, *left*), but is highly induced in multinucleated myotubes incubated in differentiation medium for 4 days (d4, *right*). Cells stained for MyHC by IF and DAPI are used to calculate the myogenic index, defined as the percentage of nuclei present in MHC-positive cells (*see* Subheading 3.3.3). We commonly score myogenic index at d4, but in some experiments the myogenic index can increase up to day 6. Scale bar, 40 μm

Fig. 2 (continued) levels, but also is found in cytoplasmic aggregates. (**a**, *lower*) High-magnification view emphasizes the presence of V5-Lem2 in cytoplasmic aggregates. This pattern occurs in nearly all V5-positive cells, regardless of the expression level. (**b**, *upper*) Lentivirus-infected cells (*arrows*) exhibit more similar cell-to-cell expression levels of V5-Lem2. Moreover, little of the protein is found in cytoplasmic aggregates. (**b**, *lower*) Higher-magnification view shows the strong localization of V5-Lem2 at the NE in the virtual absence of cytoplasmic staining. Scale bars, 40 μm (**a** and **b**, *upper*) and 5 μm (**a** and **b**, *lower*)

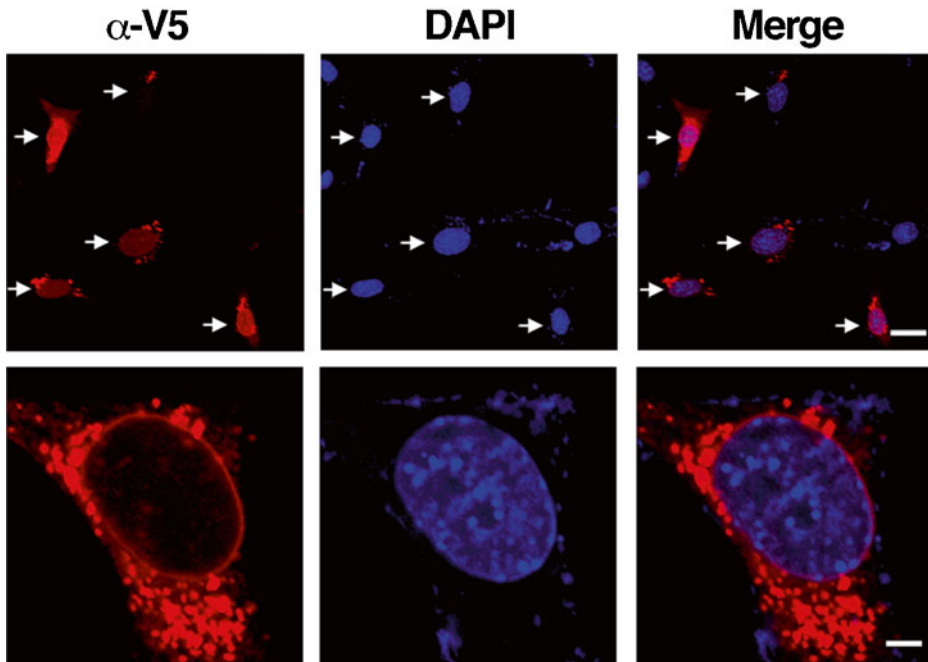
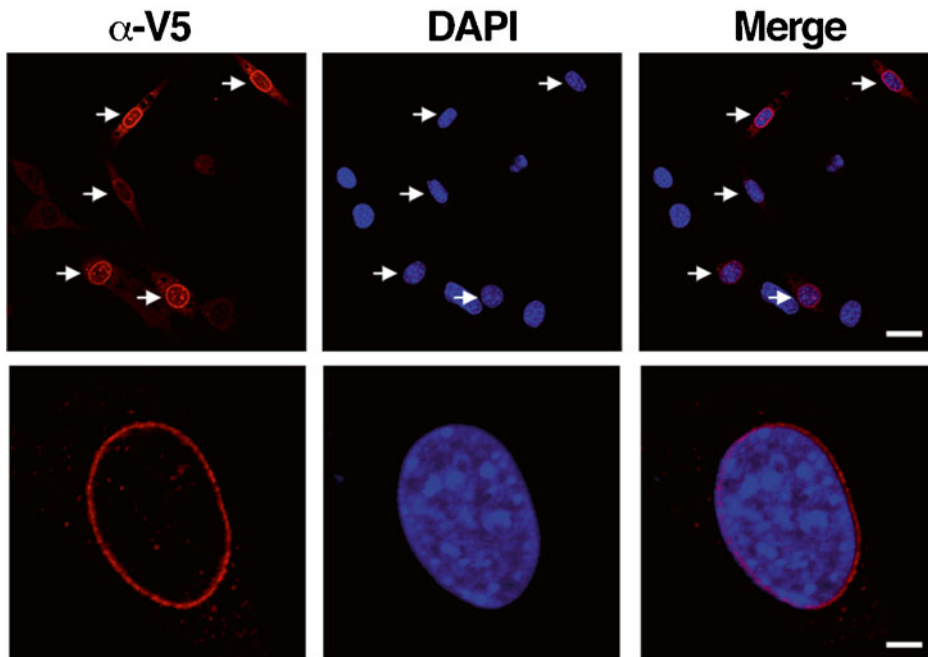
a**Lipid transfection, V5-Lem2****b****Lentiviral transduction, V5-Lem2**

Fig. 2 Comparison of lipid-mediated transfection and lentiviral-mediated gene delivery in C2C12 cells. **(a and b)** Evaluation by IF (see Subheading 3.2.2) of C2C12 cells expressing V5-Lem2 at 24 h after transfection with Lipofectamine 2000 using the manufacturer's instructions **(a)**, or at 24 h following infection with lentivirus expressing V5-Lem2 (see Subheading 3.2.1) **(b)**. **(a, upper)** Transfected V5-positive cells (arrows) exhibit a wide cell-to-cell range in the levels of V5-Lem2. The ectopic protein is localized to the NE at various

lentiviral expression yields much more uniform cell-to-cell levels of ectopic protein expression without substantial accumulation in cytoplasmic aggregates (shown in Fig. 2b for Lem2). We have obtained similar results in mouse embryo fibroblasts (MEFs) and other cell types.

Here we describe a facile strategy for carrying out structure-function analysis of nuclear lamina proteins in myoblast differentiation using C2C12 cells. Our approach involves lentivirus-mediated expression of ectopic versions of the lamina proteins in C2C12 cells [13] and isolation of bulk-selected cell populations expressing the ectopic proteins, followed by depletion of the endogenous counterparts by RNAi. Cells manipulated in this manner are then analyzed by a myoblast differentiation protocol for the ability of the mutant (or wild-type) proteins to functionally complement the loss of the endogenous proteins (Fig. 3a). An example of this is shown for Lem2 (Fig. 3b), whose depletion strongly inhibits differentiation of C2C12 cells [7]. Using the functional complementation protocol described, differentiation is fully restored by ectopic expression of wild-type Lem2 (Fig. 3b). One technical advantage of this approach is that the rapid selection/knockdown strategy allows the analysis of mutant versions of lamina proteins that might be toxic to cells in long-term culture or that can induce compensatory changes in gene expression that may obscure primary phenotypes.

In addition to using the approaches described here to study myogenic differentiation of C2C12 cells, we have implemented similar protocols to functionally dissect nuclear lamina proteins in mouse embryo fibroblasts (MEFs) in relation to aberrant regulation of ERK signaling, which is often seen with laminopathies [5]. More generally, the methods described here should be applicable to the analysis of a diverse range of cultured cell models using various functional assays, due to the wide cell tropism of lentivirus [14] and the high efficiency of siRNA delivery to most cell types.

2 Materials

2.1 Cells

1. 293T/17 packaging cells (catalog number CRL-11268) (ATCC), a clonal derivative of the 293T cell line that constitutively expresses the simian virus 40 (SV40) large T antigen. These cells are transfected efficiently and support production of high lentivirus titers.
2. C2C12 cells (catalog number CRL-1772) (ATCC), an immortalized murine myoblast line. These cells undergo efficient differentiation into multinucleated myotubes upon growth factor withdrawal [12].
3. Growth medium: Dulbecco's Modified Eagle Medium (DMEM) [+] 4.5 g/L D-glucose, [+] 110 mg/L sodium pyruvate, [-]

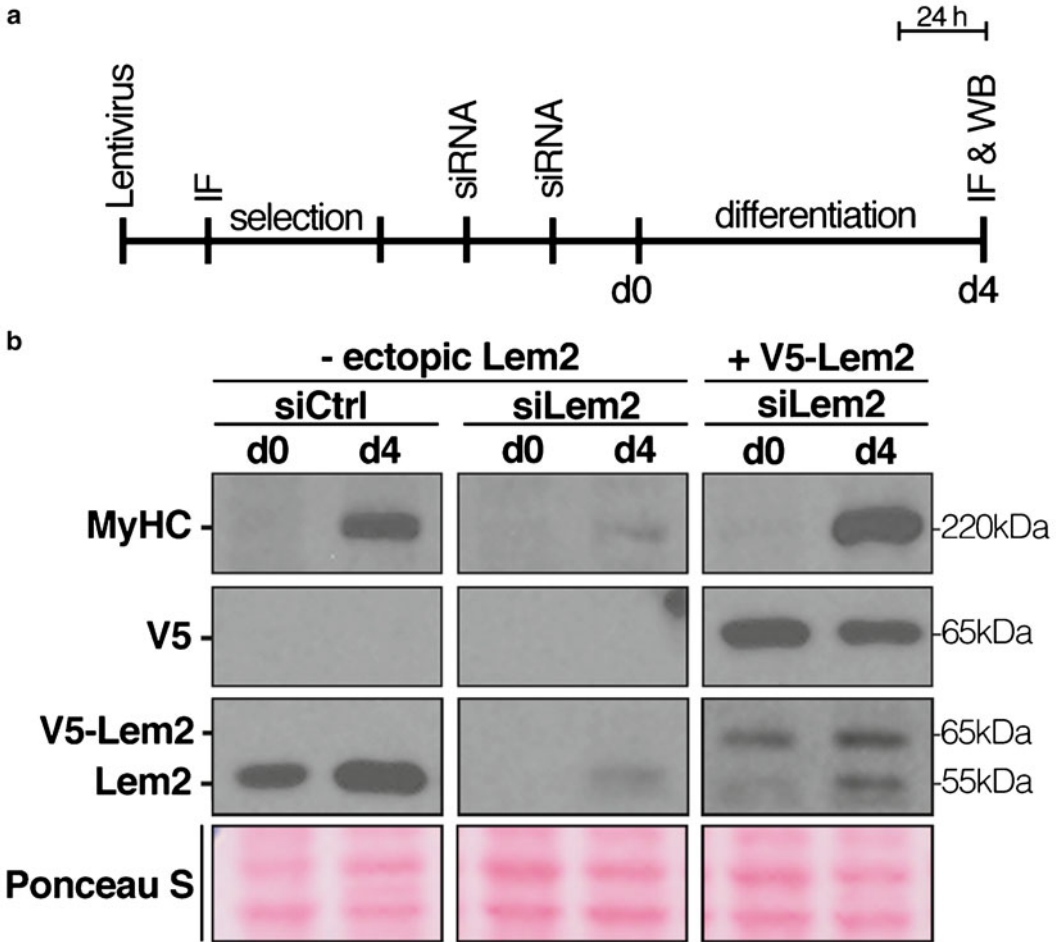


Fig. 3 Functional complementation analysis of nuclear lamina proteins in myoblast differentiation. **(a)** Schematic diagram of the experimental protocol used to analyze the effects of wild-type or mutant versions of nuclear lamina proteins on C2C12 myogenic differentiation (see Subheading 3.3). First, cells are transduced with lentivirus to express an ectopic epitope-tagged version of a nuclear lamina protein (or a mutant version thereof). Populations of virus-transduced cells are then selected by growth in antibiotic-containing medium. Next, cultures are subjected to two consecutive transfections with either a nontargeting control siRNA or with an siRNA targeting the 3' or 5' UTR of the mRNA for the endogenous nuclear lamina protein and grown to confluency (d0). Finally, after 4 days of differentiation, cells are assayed by IF and Western blotting (WB). Scale bar, 24 h. **(b)** An example of this protocol, in which expression of wild-type ectopic Lem2 is used to complement knockdown of endogenous Lem2 to support myogenic differentiation. The experiment compares non-transduced C2C12 cells ("C2C12") to C2C12 populations expressing ectopic Lem2-V5 ("C2C12-V5-Lem2"). Shown is a Western blot analysis at d0 and d4 of the differentiation protocol (see Subheading 3.3.4), using antibodies to detect the myogenic marker MyHC (upper panel), ectopically expressed Lem2 (middle panel), or both ectopic and endogenous Lem2 (lower panel). Cells transfected with nontargeting siRNA (siCtrl) or with siRNA directed against the 3' UTR of Lem2 mRNA (siLem2) are indicated. The upper panel reveals strong upregulation of MyHC in d4 control cells, indicating strong myogenic differentiation. Differentiation is strongly inhibited in cells treated with siRNA to deplete endogenous Lem2 and is rescued by expression of ectopic silencing-resistant Lem2

L-glutamine supplemented with 10 % fetal bovine serum (FBS), 1 % MEM nonessential amino acids (MEM NEAA 100×), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.

4. Differentiation medium: DMEM supplemented with 2 % donor equine serum (catalog number SH30074.02) (HyClone), 1 % MEM NEAA, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.
5. PBS (Dulbecco's Phosphate Buffered Saline): Diluted solution prepared from stock 10× PBS in sterile H₂O.
6. Plate-coating solution: 0.15 % (w/v) gelatin in sterile H₂O.

2.2 Calcium Phosphate Transfection

1. 2× HEPES-buffered saline (HBS 2×): 50 mM HEPES, 1.5 mM Na₂HPO₄, 280 mM NaCl, 10 mM KCl, and 12 mM D-glucose. Adjust separate aliquots to pH 7.05, 7.1, and 7.15, sterilize by passing through a 0.22-µm filter, and test for optimal transfection (*see Note 1*).
2. Calcium chloride: 2 M stock solution in distilled water. Pass through a 0.22-µm filter and store at 4 °C.
3. Packaging plasmids for production of third-generation lentivirus, obtained from the nonprofit plasmid repository Addgene (<http://www.addgene.org>): plasmids encoding Gag and Pol (pMDL/pRRE; catalog number 12251), Rev (pRSV-Rev; catalog number 12253), and VSV-G (pCMV-VSV-g; catalog number 8454).
4. Lentiviral transfer vector encoding gene of interest (GOI) or GFP driven by the CMV promoter, e.g., pLenti CMV-GFP Puro (catalog number 17448) (Addgene) (*see Note 2*). Lentivirus transfer vectors in which the GOI is driven by other promoters also may be used.
5. 28 mm diameter syringe filter, 0.45 µm pore SFCA membrane (e.g., catalog number 431220) (Corning Incorporated).
6. Polybrene transfection reagent (e.g., catalog number TR-1003-G) (EMD Millipore).
7. Puromycin.

2.3 Immunofluorescence Staining

1. PBS: 10 mM disodium phosphate (Na₂HPO₄), 2 mM potassium phosphate dibasic (KH₂PO₄), 137 mM NaCl, and 2.7 mM KCl; pH 7.4.
2. Fixative: 4 % paraformaldehyde solution in PBS, diluted from a 16 % stock solution. Keep in dark and use within 2 h.
3. Permeabilization buffer: 0.5 % Triton X-100 in PBS.
4. Blocking buffer: 3 % (w/v) BSA (bovine serum albumin fraction V) in 0.05 % Tween-20 in PBS. Pass through a 0.22-µm filter and store at 4 °C.

5. Mounting medium containing antifading reagent, e.g., ProLong Gold Antifade Mountant with DAPI (Life Technologies).
6. Micro cover glasses, 12-mm circle no.2.
7. Primary antibodies: mouse monoclonal anti-MyHC (skeletal myosin heavy chain) (e.g., catalog number M9850-15B) (US Biologicals) and either mouse monoclonal anti-V5 tag or rabbit polyclonal anti-V5 tag.
8. Secondary antibodies: anti-mouse, Cy3-labeled; anti-mouse, Alexa Fluor 488-labeled; anti-rabbit Cy3-labeled; or anti-rabbit Alexa Fluor 488-labeled. When dual labeling is being undertaken, it is important to use secondary antibodies with minimal cross-reactivity between mouse and rabbit.

2.4 siRNA Transfection

1. OptiMEM (reduced serum medium, [+] Hepes, [+] 2.4 g/L sodium bicarbonate, [+] L-glutamine).
2. DharmaFECT-1, transfection reagent optimized for efficient and low-toxicity small RNA delivery (Dharmacon) (*see Note 3*).
3. 5× siRNA buffer (Dharmacon).
4. Dicer-substrate siRNA duplexes. Use siRNAs directed against the 3' or 5' untranslated region (UTR) of the target mRNA and as a control, a universal nontargeting siRNA (e.g., catalog number DS NC1) (IDT) (*see Note 4*). The transduced mRNA, which contains only the coding region of the transduced protein, will be silencing resistant. Dissolve the siRNA duplexes in siRNA buffer (prepared by diluting 5× siRNA buffer stock 1:5 in sterile H₂O) to a final concentration of 20 μM (20 pmol/μL).

2.5 Western Blotting

1. Tris-glycine electrophoresis buffer: 25 mM Tris base, 192 mM glycine (electrophoresis grade), and 0.1 % SDS; pH 8.3.
2. Transfer buffer (Towbin formulation): 25 mM Tris base, 192 mM glycine, and 20 % ethanol; pH 8.3.
3. 4–20 % Tris-Glycine Mini Protein Gels, 1.5 mm, 15 wells.
4. Nitrocellulose membranes, 0.2-μm pore size.
5. Ponceau S: 0.2 % (w/v) stock solution in 5 % acetic acid. Store in the dark at room temperature.
6. SDS sample buffer: 50 mM Tris-HCl, 2 % SDS, 0.1 % bromophenol blue, and 10 % glycerol; pH 6.8. Supplement with 100 mM dithiothreitol (DTT) and cocktail of protease inhibitors, e.g., complete mini EDTA-free (catalog number 11836170001) (Roche). If using the listed cocktail, prepare a 100× stock solution of the protease inhibitor cocktail by dissolving one tablet in 100 μL of H₂O, aliquot, and store at -20 °C.
7. Pre-stained protein ladder.

8. Washing solution: 0.05 % Tween-20 in PBS (PBS-T).
9. Blocking solution: 5 % (w/v) instant nonfat dry milk in PBS-T.
10. Primary monoclonal mouse antibodies anti-V5 tag and anti-MyHC (*see* **item 7** in Subheading **2.3**) and antibodies against nuclear lamina proteins for comparison of endogenous and ectopic protein expression levels.
11. Secondary horseradish peroxidase-conjugated antibodies for mouse and rabbit antibodies listed above.
12. Chemiluminescence detection solutions for horseradish peroxidase conjugates, e.g., SuperSignal West Pico Luminol (Thermo Scientific).
13. Autoradiography film.
14. Imaging and quantification system for chemiluminescence, e.g., BioSpectrum 810 Imaging System and VisionWorksLS Image Acquisition and Analysis Software v.7.1 (UVP).

3 Methods

Lentiviruses produced by the methods described herein, which are pseudotyped with the VSV-G protein, are tropic to a wide range of cultured cells (*see* **Note 5**). Unless otherwise stated, procedures are carried out at room temperature and under sterile conditions in BL2 (biosafety level class II) cabinets. Cells are grown in a 37 °C incubator in the presence of 5 % CO₂.

3.1 Lentiviral Production by Calcium Phosphate Transfection

1. Plate 293T cells the day before transfection by splitting an 80–90 % confluent 10-cm plate into 3 × 10-cm plates, so that cells are ~80 % confluent the day of transfection. Virus produced by each 10-cm plate is sufficient to infect 6–12 3.5-cm plates of C2C12 cells, depending on the virus titer (*see* **step 10** in Subheading **3.1**).
2. The day of transfection, remove medium and add 6 mL of fresh DMEM + 10 % FBS 1 h prior to addition of the transfection mixture to the 293T cells.
3. To generate the transfection mixture for each 10-cm plate, dilute the following plasmid combinations in 350 µL H₂O in a sterile 4-mL polypropylene conical tube: 8 µg lentiviral transfer vector encoding GOI (*see* **Notes 2** and **7**), 8 µg pMDL-RRE, 6.3 µg pRSV-REV, and 3 µg pCMV-VSVg. Adjust final volume to 395 µL with H₂O. Include one transfection mixture with a transfer vector encoding GFP (LV-GFP) for verification of transfection efficiency.
4. Add 55 µL of 2 M CaCl₂ and mix well.
5. Add the plasmid/CaCl₂ mixture dropwise to 450 µL HBS 2× while vortexing vigorously. The use of a proper mixing procedure

at this point is critical for success. Incubate the mixture for 15 min at room temperature.

6. Add the transfection mixture to each plate and rock the plate gently to mix. Incubate the cells overnight (16 h).

After this step use extreme precaution while handling supernatants and materials exposed to lentivirus. While third-generation lentiviruses are self-inactivating (i.e., they can't replicate after genome integration), they can induce insertional mutagenesis. Treat all virus-contaminated tips, pipettes, plates, and discarded medium with 10 % bleach for 20 min to inactivate the virus.

7. After the overnight (16 h) incubation, check transfection efficiency in the sample producing LV-GFP using an inverted epifluorescence microscope. Approximately 80–90 % of the 293T cells should be GFP positive if the transfection is optimal.
8. Remove medium and add 6 mL of fresh DMEM+10 % FBS (*see Note 6*) to each 10-cm dish. Return to the incubator for another ~36 h. Longer culture periods will lead to extensive cell death and reduction of lentivirus yield.
9. Using a 5-mL syringe, collect the lentivirus-enriched supernatants from each 10-cm dish, and filter through a 0.45- μ m pore SFCA membrane filter for low protein binding (*see item 5* in Subheading 2.2) to remove cell debris. Keep the cleared supernatant on ice while making 0.5 mL aliquots. Store lentivirus-containing aliquots at -80°C . Since virus titer decreases with repeated freezing and thawing, using a virus aliquot only once after thawing will promote reproducibility. When necessary, viruses can be concentrated in a Beckman 55Ti rotor at 21,000 rpm for 90 min, and the viral pellet is resuspended in 100 μ L of HBS and stored at -80°C in 10 μ L aliquots.
10. Qualitatively assess the activity of the lentivirus preparation for transducing C2C12 cells using a drug selection protocol. Infect three 12-well plate wells with C2C12 cells plated the day before (~75,000 cells) with 125, 250, and 500 μ L of lentivirus supernatant diluted in growth medium and supplemented with 8 μ g/mL of polybrene. Adjust final culture volume to 1 mL. Follow the procedure described in **steps 3–6** in Subheading 3.2.1 and, after 48-h selection, determine the amount of lentivirus required to obtain ~60 % of puromycin (or other mammalian plasmid selection)-resistant cells. If quantitative determination of lentivirus titer is desired, follow one of the protocols described by Geraerts et al. [15].

3.2 Generation of Bulk-Transduced C2C12 Cell Populations

Analysis of specific nuclear lamina proteins in myoblast differentiation by functional complementation requires generation of cell populations that ectopically express a mutant or wild-type version of the protein of interest, ideally at an expression level near that of the endogenous protein (*see Note 7*). Although clones of cultured

cells expressing an ectopic protein at relatively uniform levels can be isolated after various transduction methods, C2C12 cells and primary myoblasts lose differentiation capacity with extensive passaging. We have found that populations of C2C12 cells that are transduced with lentivirus to express nuclear lamina proteins and bulk selected for antibiotic resistance express similar cell-to-cell levels of ectopic proteins for 4–6 passages after selection (Fig. 2b) and that these populations are suitable for functional complementation studies in myoblasts (Fig. 3). We have used this method to successfully analyze a spectrum of deletion mutants of Lem2 to map regions required for myoblast differentiation (e.g., Fig. 3b) and for attenuation of ERK signaling.

3.2.1 *Lentiviral Infection and Selection*

After thawing, C2C12 cells should be cultured in growth medium (*see* **item 3** in Subheading 2.1) under subconfluent conditions (less than 50 %) and passaged every day in order to avoid spontaneous differentiation. We have observed that cells should not be used for differentiation assays after passage 12–15, as differentiation efficiency may become inconsistent. Thus, many vials of cells should be frozen at initial passages to allow the use of low-passage cells for complementation experiments (*see* **Note 8**):

1. Plate C2C12 cells at a density of 10,000–15,000 cells/cm² in growth medium (~150,000 cells for a 3.5-cm dish) and incubate overnight (16 h).
2. Dilute the amount of lentivirus supernatant required to transduce 40–60 % of the cells (*see* **step 10** in Subheading 3.1) in 1 mL (final volume) of growth medium supplemented with 8 µg/mL of polybrene (*see* **Note 9**).
3. Aspirate the growth medium, wash cells with PBS, and replace with lentivirus-containing growth medium. Incubate overnight (16 h).
4. The next day the plate should be ~50 % confluent. Replace the medium with fresh DMEM + 10 % FBS, and grow the infected cultures for 24–48 h to allow expression of the antibiotic resistance gene, passaging as needed to keep cells under subconfluent conditions. At the final passage, include a sterile micro coverslip at the bottom of the dish.
5. Replace growth medium with DMEM + 10 % FBS containing the selection antibiotic to all plates, including a noninfected control plate (*see* **Note 10**).
6. Allow growth of the infected cells for the selection time needed to achieve death of cells lacking the antibiotic resistance gene. This should be 48 h for cells cultured with 2 µg/mL puromycin. Verify that 100 % toxicity is achieved in the noninfected control plate.

7. Use sterile forceps to remove the micro coverslip, wash with PBS, and process the coverslip for the detection of the ectopic protein by immunofluorescence (*see* Subheading 3.2.2) to examine the expression level and subcellular localization of the ectopic protein, which should be expressed in virtually all cells.
8. Change to fresh growth medium, and expand the cell population expressing the transduced protein to a 10-cm plate to perform the assay for functional complementation of myoblast differentiation (*see* Subheading 3.3).

3.2.2 Immunofluorescence Staining

We visualize the localization of ectopic proteins in lentivirus-transduced cells using antibodies against an epitope tag fused to the N- or C-terminus. We commonly use a V5 tag, although other tags also are suitable. All steps are performed at room temperature except for the primary and secondary antibody incubation (**steps 6 and 8**):

1. Collect the glass 12-mm micro coverslip from the dish with bulk-selected lentivirus-transduced C2C12 cells, growing at $\sim 15,000$ cells/cm² (*see* **step 7** in Subheading 3.2.1), and transfer to a clean 6-plate well.
2. Rinse the coverslip with PBS and add 2 mL of freshly prepared 4 % paraformaldehyde solution in PBS. Fix cells for 15 min.
3. Rinse the coverslips with PBS and permeabilize the cells with 0.5 % Triton X-100 in PBS for 10–15 min.
4. Perform three washes of 5 min with PBS followed by one wash with 3 % BSA in PBS for 15 min to limit nonspecific binding of the primary antibody.
5. Transfer the coverslip to a dry surface, remove excess moisture by blotting the edge, and add 50–100 μ L of anti-V5 antibody diluted 1:100 in PBS. Incubate the coverslips for 3 h using a humid chamber (*see* **Note 11**).
6. Following the primary antibody incubation, perform three washes of 5 min with PBS.
7. Incubate the coverslip with the anti-mouse Cy3-conjugated secondary antibody diluted 1:500 in PBS, and incubate the coverslips for 45 min in a light-protected humid chamber.
8. Following the incubation time, perform three washes of 10 min with PBS.
9. Remove PBS from the coverslip by blotting the edge. Cover with 20 μ L of antifade medium and remove by blotting the edge. Repeat this step, to assure complete removal of the last traces of PBS from the mounting medium.
10. Place the coverslip, cell side facing down, on a clean glass slide on top of 5–10 μ L of antifade medium. Remove excess medium

with filter paper and seal with nail polish. Allow overnight (16 h) polymerization.

11. Use an epifluorescence or confocal microscope to validate ectopic protein expression and to analyze the subcellular localization pattern of ectopic proteins. Nuclear lamina proteins, when expressed at appropriate levels (*see Note 7*), should be strongly concentrated at the NE relative to the more peripheral ER (Fig. 2b).

3.3 Functional Complementation of Myoblast Differentiation

For functional complementation studies, we prepare bulk-selected populations of C2C12 cells expressing ectopic wild-type or mutant versions of the nuclear lamina protein. The experimental groups should include the following: (1) as a positive control, C2C12 cells transfected with nontargeting siRNAs where proper myoblast differentiation is achieved (*see Note 12*); (2) as a negative control, C2C12 cells treated with Dicer-substrate siRNA directed against the 5' or 3' UTR of the mRNA to knock down the protein that causes a defect in myoblast differentiation; and (3) the functional complementation assay, C2C12 cells stably expressing ectopic wild-type or mutant versions of the nuclear lamina protein where endogenous protein is knocked down by siRNA-mediated gene silencing. This last experimental group will reveal if ectopic mutant proteins are able to functionally rescue myogenic differentiation in cells where the endogenous protein has been depleted. Rescue of differentiation by the ectopic wild-type protein validates the specificity of the siRNA. For each experimental group, set up two plates (for analysis of initial cells and terminal differentiation, d0 and d4/d6) or 6 plates (for analysis of myoblast differentiation kinetics, d0, d1, d2, d3, d4, and d6) (*see Note 13*).

3.3.1 siRNA-Mediated Knockdown of Endogenous Protein

After isolating bulk-selected cells expressing GFP or a mutant or wild-type ectopic nuclear lamina protein, we typically carry out two sequential steps of siRNA treatment to deplete the endogenous protein. Our protocol for the latter is performed by “reverse” transfection, which involves addition of the siRNA solution to a trypsinized cell suspension at the time of plating. We commonly obtain 75–90 % knockdown of various nuclear lamina proteins by this method. For each experiment, we include a sample with a nontargeting control siRNA. We initially analyze two separate siRNAs against the target protein until functional complementation by the wild-type protein is established, to rule out off-target effects of individual siRNAs. The following procedure is described for reverse siRNA transfection of a 3.5-cm dish of C2C12 cells:

1. In tube A, add 10 μL of DharmaFECT-1 transfection reagent to 100 μL of OptiMEM, and in tube B, add 25–100 pmol of an appropriate siRNA (*see Note 14*), dissolved in siRNA buffer (*see Subheading 2.4*), to 100 μL of OptiMEM (tube B), and incubate for 5 min.

2. Add the DharmaFECT-1 solution (tube A) dropwise into the siRNA solution (tube B). Mix gently by pipetting up and down, and incubate at room temperature for 15 min.
3. Trypsinize C2C12 cell populations transfected with either GFP (the control) or the V5-tagged version of the target protein, and aliquot ~200,000 cells in 1 mL growth medium in a sterile 4-mL polypropylene conical tube.
4. Dropwise add the DharmaFECT/siRNA mixture to the cells and mix gently. After 10 min seed the cells into a 3.5-cm plate and incubate overnight (16 h) in 2 mL (final volume) DMEM + 10 % FBS.
5. The next morning, cells should be ~70 % confluent. Aspirate the medium, wash with PBS, and add 2 mL of fresh growth medium. Allow cells to grow for 8 h.
6. Perform a second siRNA reverse transfection using the same amount of siRNA as in the first transfection. Replate the cells to a 3.5-cm dish with a coverslip located at the bottom, both pre-coated with 0.15 % gelatin (*see Note 15*), and incubate overnight (16 h).
7. The next morning cells should be ~70 % confluent. Aspirate the medium, wash with PBS, and add 2 mL of fresh growth medium.
8. Incubate the plates for 24–48 h until the cells reach confluency. Subsequently carry out the myoblast differentiation assay (*see Subheading 3.3.2*).

3.3.2 Myoblast Differentiation Assay

1. Beginning with siRNA-transfected cells that are grown to confluency (*see step 8* in Subheading 3.3.1), remove the growth medium and replace with differentiation medium (*see item 4* in Subheading 2.1). Cells are considered to be at differentiation day 0 (d0).
2. Using sterile forceps, collect d0 coverslips, rinse with PBS, and fix with 4 % paraformaldehyde in PBS. Store at 4 °C in PBS until used for myogenic index analysis by immunofluorescence (*see Subheading 3.3.3*). Harvest the remaining cells from the 3.5-cm dish for Western blotting (*see Subheading 3.3.4*).
3. Allow cells to differentiate for 4 days (d4) (*see Note 16*). Under the phase-contrast microscope, elongated multinucleated myotubes should be readily visible in C2C12 cells treated with nontargeting control siRNA.
4. Collect d4 coverslips, rinse with PBS, and fix with 4 % paraformaldehyde, and process d0 and d4 coverslips for immunofluorescence staining of MyHC to determine the myogenic index of each experimental group (*see Subheading 3.3.3*). Harvest the remaining cells from the 3.5-cm dish for Western blotting (*see Subheading 3.3.4*).

3.3.3 Assessment of Myogenic Differentiation by Immunofluorescence

The myogenic index, which is a morphological parameter of differentiation, is defined as the fraction of nuclei residing in MyHC-positive cells. Coverslips collected on d0 and d4 are processed to identify cells expressing the skeletal muscle protein MyHC by immunofluorescence and to monitor nuclei by DAPI staining (*see* Subheading 3.2.2). Use an inverted epifluorescence or confocal microscope to record 3–4 micrographs (~100 cells per image) and use image analysis software (i.e., ImageJ, CellProfiler, AxioVision, Zen, etc.) for measurement of the myogenic index in the various cell populations. These values will allow evaluation of how knock-down of a protein of interest affects the differentiation capacity of cells and whether the ectopic protein functionally complements the knockdown by restoring the myogenic index to levels found in “control” cultures, i.e., C2C12 cultures treated with nontargeting control siRNA. If the myogenic index of control cultures is lower than 30 %, discard the lentivirally transduced cultures, and start the protocol with another low-passage vial of C2C12 cells.

3.3.4 Assessment of Myogenic Differentiation and Knockdown Efficiency by Western Blotting

The myogenic differentiation of C2C12 cells also can be assessed by Western blotting. Upregulation of the skeletal muscle protein MyHC at day 4 commonly is used to assess differentiation (Fig. 3b). In addition, the kinetics of differentiation can be determined by evaluating the expression of myogenin and MRF4 (Myf6), muscle-specific transcription factors that are induced at days 1–2 in the differentiation program. Finally, the knockdown of the endogenous nuclear lamina protein by siRNA (*see* Subheading 3.3.1) and the expression level of ectopic epitope-tagged protein relative to endogenous protein should be determined by Western blotting.

1. After removal of the coverslip, ~80 % of the cells remain in the plate. Wash twice with PBS and add 200 μ L of SDS sample buffer (*see* item 6 in Subheading 2.5). Scrape off the cells from the dish and transfer to a microfuge tube. Incubate the extract in a 95 °C heating block for 10 min. Centrifuge the tubes at 13,000 rpm for 10 min, and load 10 μ L of each sample (~15,000 cell equivalents) on 4–20 % Tris-Glycine Mini Protein Gels. Include a lane with 7 μ L of pre-stained molecular weight protein ladder.
2. Perform polyacrylamide gel electrophoresis (PAGE) to separate proteins by running the gel for ~2 h at 130 V in Tris-glycine electrophoresis buffer.
3. Transfer proteins from the polyacrylamide gel after electrophoresis to a nitrocellulose membrane using a wet transfer apparatus at 0.4A for 1.5 h.
4. Rinse the membrane with H₂O and stain the immobilized proteins in the membrane with 0.2 % Ponceau S for 2 min. Rinse membrane with H₂O twice to remove excess dye. Scan the membranes for your records.

5. Block membranes with 5 % instant nonfat dry milk blocking solution for 30 min.
6. Rinse twice with PBS-T and incubate with the primary antibodies (*see item 10* in Subheading 2.5) diluted 1:1000 in PBS-T during 2 h at room temperature or overnight (16 h) at 4 °C with gentle rocking.
7. Wash the membranes extensively with PBS-T, and incubate with the secondary HRP-conjugated antibodies (*see item 11* in Subheading 2.5) diluted 1:5000 in PBS-T for 45 min at room temperature.
8. After several washes with PBS-T, incubate the membrane with ECL substrate for 60 s, and, after removal of liquid excess, expose the membrane to X-ray film to collect an image with an automated image analyzer such as the BioSpectrum 810 Imaging System from UPV.

4 Notes

1. Although optimal transfection should occur at pH 7.05, HBS 2× may require pH optimization. Start by adjusting the pH of HBS 2× (0.2 L) to 7.0 and split into 50 mL aliquots. Titrate separate aliquots to pH 7.05, pH 7.1, and pH 7.15. Test each of the four solutions (pH 7.0–7.15) by transfecting a lentiviral transfer vector encoding GFP using the calcium phosphate protocol (*see* Subheading 3.1) into 293T cells. The HBS 2× that gives the most efficient transfection should be aliquoted and stored at –20 °C.
2. To easily subclone the GOI in the pLenti CMV-GFP Puro vector, use BamHI and SalI restriction sites. Lentivirus transfer vectors in which the GOI is driven by other promoters also can be used (*see Note 7*).
3. GE Dharmacon offers four specialized DharmaFECT formulations. The most suitable transfection reagents for siRNA delivery in C2C12 cells are DharmaFECT-1 and DharmaFECT-3, based on delivery of siGLO Green Transfection Indicator (Dharmacon).
4. 27-mer Dicer-substrate siRNAs can be designed using RNAi design tools available from different biotechnology companies (i.e., Integrated DNA Technologies, using the RNAi design tool at <http://www.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx>). These siRNAs mimic endogenous Dicer-processed products and therefore are directly incorporated to the multiprotein RNA-inducing silencing complex (RISC) [16] but are sufficiently small to avoid inducing the interferon response that causes global inhibition of mRNA translation and mRNA degradation [17].

5. Lentiviral tropism is due to the ability of the viral envelope protein to interact with receptors at the target cell surface. The VSV-G envelope protein confers broad tropism over a range of species and cell types. Cronin et al. [18] describe different envelopes available and their impact on lentiviral tropism. For C2C12 cells, 1 mL of lentiviral supernatant diluted with 1 mL of growth medium typically yields ~40–50 % transduction efficiency.
6. During the production stage (*see step 8* in Subheading 3.1), culturing transfected 293T cells in DMEM with a lower percentage of FBS (i.e., 2–5 %) can increase the yield of lentivirus. If this variation is introduced to the protocol, adjust the FBS concentration to 10 % when diluting the lentivirus-containing supernatant with growth medium for cell infection.
7. To regulate ectopic gene expression, the use of different promoters (i.e., EF1a, PGK, or UBC) can be explored. In most cells the order of promoter strength is like CMV > EF1a > PGK > UBC. The following vectors that drive expression of the GOI under the control of the alternative promoters EF1a, PGK, and UBC, respectively, are available from the nonprofit plasmid repository Addgene (<http://www.addgene.org>): pEGIP (catalog number 26777), pLenti PGK GFP Puro (catalog number 19070), and Ubc.Luc.IRES.Puro (catalog number 33307). Alternatively, lentivirus transfer vectors containing inducible promoters such as the tetracycline ON system can be used to tune the expression of the ectopic protein. These vectors contain a tet-response element (TRE) upstream the promoter region that is recognized by the reverse tetracycline-controlled transactivator (rtTA). The rtTA binds to the TRE only in the presence of doxycycline to activate transcription. A single lentiviral transfer vector encoding the puromycin resistance gene, the rtTA, and the TRE3G promoter that drives a tightly regulated expression of the GOI is commercially available (Lenti-X Tet-One Inducible Expression System, Clontech Laboratories).
8. To quickly make stable populations of target cells, lentiviral transfer vectors must have a suitable antibiotic selectable marker, such as the puromycin or blasticidin resistance gene, which causes cell killing within several days (*see Note 10*). As an alternative to antibiotic resistance selection, lentiviral transfer vectors with a dual promoter for coexpression of the GOI and GFP can be used to isolate GFP-positive cells by fluorescence-activated cell sorting (FACS), for expansion of transduced cell populations with different levels of expression.
9. Polybrene is a cationic polymer that increases the efficiency of lentivirus infection by neutralizing negative charges and facilitating the fusion of the virus membrane and the plasma membrane of the host cell [19].

10. Antibiotic selection of any particular target cell type requires determination of the appropriate antibiotic concentration by performing a kill curve. For C2C12 cells, selection medium containing 2 $\mu\text{g}/\text{mL}$ puromycin or 20 $\mu\text{g}/\text{mL}$ blasticidin achieves 100 % cellular toxicity after 48 h of incubation.
11. Include the following immunofluorescence controls to validate the quality of antibodies and appropriate dilution: (1) by incubation with secondary antibody to evaluate fluorescence background signal and (2) by labeling of non-transfected cells to evaluate nonspecific binding of the primary antibody.
12. We typically analyze C2C12 cells that are lentivirally transduced with GFP in parallel as a control to validate that the lentivirus infection and selection are not deleterious to differentiation.
13. Differentiation kinetics can be measured by evaluation of the expression of early myogenic markers, myogenin and MRF4 (Myf6). Analysis involves samples each day during the course of the myoblast differentiation (d0, d1, d2, d3, and d4) to reveal the rate-limiting day.
14. Selecting the best siRNA sequences for knockdown should be empirically determined by initially comparing 3–4 different siRNAs. Even with the most potent siRNAs, the amount of siRNA needed to achieve effective knockdown will vary for different proteins, depending on the protein and mRNA stability. It is important to perform a titration experiment to determine this. Using a lower concentration of siRNA is more cost-effective and reduces the chances for off-target effects. We usually test a range of 25, 50, 75, and 100 pmol of siRNA for 200,000 cells. Whether one or two reverse transfections with siRNA are needed to achieve ~80 % knockdown also should be empirically determined. In the particular cases of Lem2 and emerin, two sequential treatments with 100 pmol siRNA on consecutive days yield 80–90 % knockdown. By contrast, maximum knockdown of Net37 is achieved with a single 50-pmol siRNA treatment.
15. For gelatin-coating plates, prepare a 1.5 % stock solution of gelatin in H_2O under sterile conditions. Dilute 1:10 in H_2O and cover each 3.5-cm dish containing a coverslip with 2 mL of 0.15 % gelatin. Incubate at 37 °C for 30 min. Aspirate and air-dry the plates at room temperature under UVA light for 30 min. Sealed plates can be stored at 4 °C.
16. When, after siRNA-mediated silencing of the nuclear lamina protein, myoblast differentiation is blocked (absence of MyHC signal by d4), further validation of C2C12 terminal differentiation by d6 should be performed. Also, differentiation kinetics should be measured by collecting samples each day (d0, d1, d2, d3, and d4) to evaluate the onset expression of early myogenic markers (i.e., myogenin and MRF4/Myf6).

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