Lamins A and C but Not Lamin B1 Regulate Nuclear Mechanics

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Mutations in the nuclear envelope proteins lamins A and C cause a broad variety of human diseases, including Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy, and Hutchinson-Gilford progeria syndrome. Cells lacking lamins A and C have reduced nuclear stiffness and increased nuclear fragility, leading to increased cell death under mechanical strain and suggesting a potential mechanism for disease. Here, we investigated the contribution of major lamin subtypes (lamins A, C, and B1) to nuclear mechanics by analyzing nuclear shape, nuclear dynamics over time, nuclear deformations under strain, and cell viability under prolonged mechanical stimulation in cells lacking both lamins A and C, cells lacking only lamin A (i.e. “lamin C-only” cells), cells lacking wild-type lamin B1, and wild-type cells. Lamin A/C-deficient cells exhibited increased numbers of misshapen nuclei and had severely reduced nuclear stiffness and decreased cell viability under strain. Lamin C-only cells had slightly abnormal nuclear shape and mildly reduced nuclear stiffness but no decrease in cell viability under strain. Interestingly, lamin B1-deficient cells exhibited normal nuclear mechanics despite having a significantly increased frequency of nuclear blebs. Our study indicates that lamins A and C are important contributors to the mechanical stiffness of nuclei, whereas lamin B1 contributes to nuclear integrity but not stiffness.

Lamins are type V intermediate filament proteins that form the nuclear lamina, a filamentous network underlying the inner nuclear membrane of eukaryotic cells. Lamins form stable structures in the nuclear lamina and the nucleoplasm, determine nuclear shape and size, resist nuclear deformation, and position nuclear pore complexes (reviewed in Refs. 1–3). In addition, lamins recruit and anchor, either directly or indirectly, several nuclear envelope proteins (e.g. nesprin-1α, emerin, and the lamin B receptor) to the inner nuclear membrane (3).

Mammalian cells express two types of lamins, the A and B types. Both share a common structural organization: a globular N-terminal domain separated from a larger C-terminal globular domain by a central helical rod domain that allows lamins to form parallel coiled-coil dimers, which in turn assemble into stable strings and higher order networks. Lamins A and C, the major A-type lamins, are alternatively spliced isoforms of a single gene, LMNA. The expression of A-type lamins is developmentally regulated, beginning midway through embryonic development (4). A-type lamins are expressed in most but not all differentiated cells (5). B-type lamins (lamins B1 and B2) are encoded by separate genes, LMNB1 and LMNB2, respectively (6). Unlike A-type lamins, B-type lamins are expressed in all cells and throughout development (7, 8), although it is not clear if they are always coexpressed at equivalent levels in the same cell.2

Mutations in the A-type lamins and associated nuclear envelope proteins cause at least 10 distinct human diseases (laminopathies), including Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy with conduction system disease, Duchenne-type familial partial lipodystrophy, and Hutchinson-Gilford progeria syndrome (reviewed in Refs. 9–13). Mice lacking both lamins A and C manifest severe growth retardation as well as skeletal and cardiac muscle dystrophy, and they die by 4–6 weeks of age (14, 15). In contrast, lamin C-deficient mice are entirely healthy (16). No human diseases have yet been linked to mutations in B-type lamins, raising the possibility that the loss of B-type lamins is either inconsequential or lethal during development. Gene silencing experiments in cultured mammalian cells with small interfering RNAs have suggested that lamins B1 and B2 are essential for cell growth and cell viability (17). Genetically modified mice lacking wild-type lamin B1 have bone and lung abnormalities during development and die shortly after birth (18).

Mechanisms underlying the panoply of human laminopathies (particularly how entirely different tissues are affected in different diseases) are poorly understood. Two hypotheses, neither mutually exclusive, have emerged to explain how mutations in lamin proteins could cause such diverse disease pheno-
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The structural hypothesis is compelling, given the obvious scaffolding role of the nuclear lamina and the relationship, at least in some studies, between misshapen nuclei and disease phenotypes in mice (14–16, 22, 23). However, there is a paucity of data on the structural properties of the nuclear lamina and how different lamins might contribute to the structural properties of the nuclear lamina. In this study, we have taken advantage of the existence of genetically modified mice (and fibroblast and myoblast cell lines derived from them) to understand the roles of specific lamin proteins in nuclear mechanical stiffness and fragility and in cell viability.

**Experimental Procedures**

**Cells—**Lmna-deficient (Lmna\(^{-/-}\)), Lmna heterozygous (Lmna\(^{+/-}\)), and wild-type (Lmna\(^{+/+}\)) mouse embryonic fibroblasts (MEFs)\(^3\) were cultured from embryos derived from Lmna\(^{+/-}\) intercrosses (14). Similarly, lamin C-only (Lmnc\(^{+/-}\)) and wild-type control MEFs were derived from the offspring of Lmna\(^{+/-}\) intercrosses. Lmna\(^{+/-}\) MEFs were obtained by intercrossing Lmna\(^{+/-}\) and Lmna\(^{+/-}\) mice. Cells lacking wild-type lamin B1 (Lmb1\(^{+/-}\)) express a fusion protein consisting of roughly the first half of lamin B1 and βgeo; the lamin B1 fusion protein lacks several crucial functional domains of lamin B1 (e.g. a large portion of the rod domain and the C-terminal globular domain, including the CAAX (where A is an aliphatic amino acid) motif that triggers farnesylation) (18). For each genotype, we used at least two independently derived lines of MEFs. MEFs were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum (HyClone), nonessential amino acids, 2-mercaptoethanol, and penicillin/streptomycin. Experiments were performed both on primary MEFs and on MEFs that had been immortalized by repeated passages. Primary and immortalized MEFs invariably showed the same phenotypes, but cell-to-cell variations were smaller in the immortalized cell lines. Accordingly, unless specified otherwise, the experiments were performed with immortalized MEFs. Skeletal myoblasts were derived from the fore and hind limbs of 4–6-week-old Lmna\(^{+/-}\) and Lmna\(^{-/-}\) mice as described previously (24). Myoblasts were cultured on gelatin-coated dishes in Ham’s F-10 medium (Invitrogen) supplemented with 1.26 mM Ca\(^{2+}\), 20% horse serum, 10 ng/ml basic fibroblast growth factor (R&D Systems), and penicillin/streptonycin. Proliferating myoblasts were refed daily with fresh growth medium. Differentiation was induced by growth factor withdrawal.

**Western Analysis of Nuclear Proteins—**Cells were grown to confluency and lysed in radioimmune precipitation assay buffer supplemented with 300 mM NaCl, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (all from Sigma) or alternatively in 9 mM urea buffer supplemented with 10 mM Tris-HCl, 0.2% 2-mercaptoethanol, 10 μM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture. Protein extracts were size-fractionated on 4–12% BisTris gels (Invitrogen) with MOPS running buffer; transferred onto polyvinylidene difluoride membranes; and incubated with antibodies against lamin A and C (catalog no. sc-6215, Santa Cruz Biotechnology, Inc.), lamin B1 (catalog no. sc-6217, Santa Cruz Biotechnology, Inc.), lamin B2 (rabbit polyclonal antibody kindly provided by Dr. Larry Gerace), α-tubulin (catalog no. sc-5286, Santa Cruz Biotechnology, Inc.), and actin (catalog no. A-2066, Sigma).

**Nuclear Shape Analysis—**To quantify the fraction of irregularly shaped nuclei or nuclei containing blebs, MEFs at various passages were incubated for 15 min with 1 μg/ml Hoechst 33342 nuclear stain (Molecular Probes) and washed with Hanks’ buffered saline solution (HBSS; Invitrogen). For each passage, fluorescent images of ~100–400 randomly selected nuclei were acquired at ×20 magnification on an Olympus IX-70 microscope with a digital camera and stored for subsequent image analysis (19). Nuclei were scored as normal, as irregularly shaped (deviation from an oval or spherical shape), or as containing blebs. These determinations were made by an observer blinded to genotype. For quantitative nuclear morphometric analyses, the nuclear cross-sectional area and perimeter were measured in midsections of the fluorescently labeled nuclei with custom-written MATLAB software and used to compute the nuclear contour ratio (4π×area/perimeter\(^2\)) (25). The contour ratio reaches a maximum value of 1 for a circle and decreases with increasingly convoluted nuclear shapes. In addition, elliptic Fourier analysis was used for a more detailed analysis of nuclear contour using custom-written MATLAB software to automatically trace the outline of fluorescently labeled nuclei and to compute the first 20 elliptic harmonics, where each single elliptic harmonic can be geometrically visualized as a pair of orthogonal semiaxes (19, 26). For the nuclear shape analysis, we defined the elliptic Fourier coefficient (EFC) ratio as the ratio of the sum of the first major and minor semiaxes to the sum of the higher order semiaxes as a metric of irregular nuclear shape, where the first-order harmonic coefficients can be visualized as describing an ellipsoidal shape and higher order harmonic coefficients are necessary to fit more convoluted outlines.

**Immunofluorescence Microscopy—**Cells were plated on LabTek chamber slides (Nunc), fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. After blocking, cells were incubated with primary antibodies (anti-lamin B, anti-lamin A/C, anti-emerin (Abcam), and anti-β-tubulin (Sigma)) or Oregon Green 488-phalloidin (Molecular Probes), followed by incubation with secondary antibodies conjugated to Cy3 (Sigma) or Alexa Fluor 488 (Molecular Probes). Subsequently,

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\(^3\)The abbreviations used are: MEFs, mouse embryonic fibroblasts; DMEM, Dulbecco’s modified Eagle’s medium; BiTris, 2-(bis(2-hydroxyethyl)-amino)-2-(hydroxymethyl)propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; HBSS, Hanks’ buffered saline solution; EFC, elliptic Fourier coefficient; RFP, red fluorescent protein; GFP, green fluorescent protein.
fixed cells were imaged on an Olympus IX-70 microscope at ×40 (numerical aperture of 1.15) or ×63 (numerical aperture of 1.4) magnification on a Zeiss LSM510 laser scanning confocal microscope. Perinuclear cytoskeletal structure was scored by an observer blinded for genotype as either normal or abnormal (e.g. fragmented actin filaments or sparse microtubule network).

Time-lapse Experiments—To quantify dynamic changes in nuclear shape, we performed time-lapse video microscopy (19). Cells were plated on 35-mm polystyrene cell culture dishes (Corning) and grown in DMEM containing 10% fetal calf serum at 37 °C for at least 24 h prior to the start of experiments. Subsequently, culture dishes were sealed with Parafilm M and placed at room temperature on the microscope stage. After a brief equilibration period, images were automatically acquired every 5 min for a minimum of 8 h, 20 min (100 frames), at ×20 magnification with a digital CCD camera. Continued cell viability was confirmed by monitoring the cells for 24 h. Nuclear shape changes, rotation, and translation were analyzed by tracking the centroid positions of three to six nucleoli for each nucleus with custom-written MATLAB software. For each frame, the linear conformal image transformation that best mapped the current centroid positions to the original positions was computed, minimizing the least-square error. The linear conformal transformations can account for a combination of translation, rotation, and scaling, and this method preserves the conformal transformations can account for a combination of translation, rotation, and scaling, and this method preserves the relative position of objects to each other. The deviation from the best fit (i.e., the error between the least-square fit transformation and the actual nucleolus positions) was used as a measure of nuclear deformation, as it describes the extent of nuclear deformation from the initial shape independent of absolute nuclear movement or uniform changes in size (see supplemental Videos 1–4). Nucleoli that fused over time or cells that died or underwent mitosis during or immediately following the observation period were excluded from the analysis. We analyzed at least 23 nuclei of each cell type, and we computed for each nucleus the time-averaged deformation and the time-averaged and maximum change in size.

Nuclear Strain Experiments—Nuclear strain studies were performed as described previously (19, 20). In brief, cells were plated on fibronectin-coated silicone membranes in DMEM supplemented with 10% fetal calf serum, followed by serum starvation for 48 h in DMEM containing insulin, transferrin, and selenium supplements (Sigma) to minimize the number of mitotic nuclei. Preceding the strain experiments, cells were incubated with Hoechst 33342 nuclear stain (final concentration of 1 μg/ml) in DMEM containing insulin, transferrin, and selenium supplements for 15 min and subsequently kept in HBSS. Membranes were placed on a custom-made strain device, and uniform biaxial strain was applied to the silicone membrane. Membrane and nuclear strains were computed based on bright-field and fluorescent images acquired before, during, and after strain application with a custom image analysis algorithm. Normalized nuclear strain was defined as the ratio of nuclear strain to membrane strain to compensate for small variations in applied membrane strain. The percent membrane strain was 4.76 ± 0.37 (mean ± S.D.). The myoblasts experiments were carried out analogously to the fibroblast experiments. Myoblasts were plated on fibronectin-coated silicone membranes and allowed to differentiate for 5 days prior to the start of the experiments, resulting in large multinucleated myotubes (see Fig. 7).

Lmna<sup>−/−</sup> Rescue Experiments—Lmna<sup>−/−</sup> MEFs were transfected with expression plasmids coding for prelamin A-red fluorescent protein (RFP), green fluorescent protein (GFP)-lamin B1, or GFP-lamin C with GeneJammer transfection reagent (Stratagene). The next day, cells were replated on fibronectin-coated silicone membranes and, following 48 h of serum starvation, used for nuclear strain experiments. Additional aliquots of transfected cells were replated on 35-mm polystyrene dishes or LabTek chamber slides and imaged 1–3 days after transfection.

Cell Viability and Apoptosis Assays—Experiments to measure cell viability under prolonged strain application were carried out as described previously (19, 20). Cells were plated on fibronectin-coated silicone membranes and maintained in full medium for 1–3 days. Following 24 h of cyclic biaxial strain (1 Hz, 10% strain), cells were incubated for 15 min with propidium iodide (final concentration of 1 μg/ml; Sigma). The cells and culture medium were collected, washed once with HBSS, and resuspended in HBSS. Each sample was divided into two equal parts to measure cell death (propidium iodide uptake) and apoptosis (DNA content analysis). One part was immediately analyzed for propidium iodide uptake with a flow cytometer (Cytomics FC500, Beckman Coulter), counting 30,000–50,000 events in each group. Thresholds for propidium iodide incorporation were determined based on a negative control (no propidium iodide staining) and a positive control (propidium iodide staining after permeabilization with 50% ethanol). The other cell fraction was fixed in 80% ethanol and stored at −20 °C. Samples were subsequently spun down, resuspended in calcium- and magnesium-free Dulbecco’s phosphate-buffered saline (Invitrogen), treated with ribonuclease A (Sigma) for 30 min, and stained for 15 min with propidium iodide (final concentration of 100 μg/ml). DNA content was measured with a flow cytometer (counting 30,000–50,000 events/sample), and apoptotic cells were identified as those with sub-G<sub>1</sub> DNA content (27).

Image Acquisition and Manipulation—Phase-contrast and fluorescent images were acquired with a CoolSNAP<sub>HiQ</sub> digital CCD camera (Photometrics) mounted on an Olympus IX-70 inverted microscope with Image-Pro image acquisition software (Media Cybernetics). Nuclear shape experiments and time-lapse studies were imaged with an Olympus LPlanFl×20 phase-contrast objective (numerical aperture of 0.40), whereas nuclear strain experiments were conducted with an Olympus LPlanFl×60 objective (numerical aperture of 0.70). Cells were kept in HBSS during imaging, except for time-lapse experiments, in which the cells were kept in complete medium. All experiments were carried out at room temperature.

Statistical Analysis—All experiments were performed at least three times. Data are expressed as the mean ± S.E. Statistical analysis was performed with Prism 3.0 and InStat software (GraphPad Software). The data were analyzed by unpaired Student’s t test (allowing different S.D. values) for comparison
between two groups; by one-way analysis of variance (followed by Tukey’s multiple comparison test) for three or more groups; or, in cases of non-gaussian distribution, by the Mann-Whitney or Kruskal-Wallis test (the latter when comparing more than two groups, always with Dunn’s multiple comparison post-test). For nuclear shape analyses, we compared mean values from at least three different passages (counting ~200 nuclei each) by one-way analysis of variance, followed by Tukey’s multiple comparison test for some comparisons (e.g. Lmna\(^{+/+}\) versus Lmna\(^{-/-}\) and Lmna\(^{LCO/LCO}\) versus Lmna\(^{-/-+}\) versus Lmna\(^{+/+}\)), whereas we applied a paired t test for comparisons of Lmnb1\(^{+/+}\) and Lmnb1\(^{+/+}\). Histograms for quantitative nuclear shape parameters were based on pooled results from several passage numbers, and median values were compared by non-parametric one-way analysis of variance for certain comparisons (Lmna\(^{-/-}\) versus Lmna\(^{+/+}\).
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A

B

C

D

E

F



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versus Lmna$^{-/-}$ and Lmna$^{LCO/LCO}$ versus Lmna$^{-/-}$) and by the Mann-Whitney test for others (Lmnb1$^{-/-}$ versus Lmnb1$^{1/2}$). Multiple regression analysis was carried out on 16 of the 21 different cell lines for which nuclear strain results and Western analysis results were available (supplemental Table 1). Normalized nuclear strain was used as the outcome, and the expression levels of lamins A, B1, B2, and C relative to levels in wild-type cells were used as the variables. Even though multicollinearity analysis showed that the variables were independent of each other, we tested only regression models that used a single variable (e.g. lamin A) at a time. For all experiments, a two-tailed $p$ value of $<0.05$ was considered significant.

RESULTS

Loss of Specific Lamins Does Not Affect Protein Levels of Other Lamins—We analyzed the function of specific lamins in nuclear shape, stability, and stiffness in wild-type MEFs and in MEFs lacking lamin A (designated lamin C-only or Lmna$^{LCO/LCO}$ cells) (16), in MEFs deficient in both lamins A and C (Lmna$^{-/-}$) (14), and in MEFs expressing a severely truncated mutant form of lamin B1 (Lmnb1$^{1/2}$) that lacks several crucial functional domains of lamin B1 (a large portion of the rod domain and the C-terminal globular domain with the CAAX motif that triggers farnesylation) (18). To determine whether the loss of specific lamins leads to compensatory up-regulation of the expression of other lamins, we performed Western analysis on whole cell lysates (Fig. 1). As expected, Lmna$^{-/-}$ MEFs lacked all A-type lamins, whereas Lmna$^{+/+}$ MEFs had reduced levels of lamins A and C (Fig. 1A). Expression of B-type lamins was not significantly perturbed in Lmna$^{-/-}$ or Lmna$^{+/+}$ MEFs. Lmna$^{LCO/LCO}$ cells did not express lamin A but expressed increased levels of lamin C (Fig. 1B). Interestingly, Lmna$^{LCO/LCO}$ MEFs had significantly increased expression of lamin B2 and showed a trend toward increased levels of lamin B1 (Fig. 1B). In Lmnb1$^{1/2}$ MEFs, lamin B1 was absent, as expected, whereas expression of lamins A, C, and B2 appeared to be normal (Fig. 1C). We did observe some variability in lamin protein levels in different cell lines of a single genotype, but this type of variability is known to occur with differences in cell cycle or quiescence state (28). Actin expression was not significantly different between any of the cell lines (data not shown).

Specific Lamin Deficiencies Lead to Distinct Defects in Nuclear Morphology—Fibroblasts from laminopathy patients often have abnormally shaped nuclei and nuclear blebbing (29–31); irregular nuclear morphology has also been reported in Lmna$^{-/-}$ and Lmnb1$^{1/2}$ MEFs (14, 18). To define the qualitative and quantitative effects of specific lamin deficiencies on nuclear morphology, we analyzed nuclear shape in Lmna$^{-/-}$, Lmna$^{LCO/LCO}$, and Lmnb1$^{1/2}$ MEFs at various passages. Nuclei were classified as being normal, having irregular shape, or having nuclear blebs with chromatin protruding from the nucleus. Some Lmna$^{-/-}$ and Lmna$^{LCO/LCO}$ MEFs had irregularly shaped nuclei (Fig. 2A); however, the fraction of abnormally shaped nuclei was lower in Lmna$^{LCO/LCO}$ cells than in Lmna$^{-/-}$ cells (Fig. 2B). The nuclear morphology of Lmna$^{+/+}$ and Lmnb1$^{1/2}$ fibroblasts was normal (Fig. 2B). Lmnb1$^{1/2}$ MEFs had an increased fraction of nuclear blebs ($p < 0.0001$) but otherwise had smooth and normally shaped nuclei (Fig. 2, A and B).

To provide a more quantitative analysis of nuclear morphology, we computed two characteristic shape parameters based on the nuclear contour and cross-sectional area. Lmna$^{-/-}$ MEFs had a decreased contour ratio compared with wild-type cells ($p < 0.001$) (Fig. 2C). The relative frequency distribution showed a wide range of contour ratios for Lmna$^{-/-}$ cells, with few cells reaching normal values and a large proportion of cells with small nuclear contour ratio values (Fig. 2D). Lmna$^{LCO/LCO}$ MEFs also had decreased contour ratios compared with wild-type cells ($p < 0.05$); however, the differences between Lmna$^{LCO/LCO}$ MEFs and wild-type controls were less dramatic. Lmnb1$^{1/2}$ MEFs did not have significantly different contour ratios compared with wild-type cells based on the average values from six independent experiments, but there were small but significant reductions in the contour ratio when analyzing data from individual nuclei ($\sim\! 1300$ nuclei each for Lmnb1$^{1/2}$ and Lmnb1$^{1/2}$ cells) instead of the average values from each independent experiment. This observation was confirmed in the frequency distributions, which revealed an elevated
FIGURE 3. Nuclear shape stability is decreased in Lmna<sup>−/−</sup> and Lmna<sup>LCO/LCO</sup> nuclei. MEFs were imaged every 5 min for a period of 8 h, 20 min. Dynamic changes in nuclear shape were quantified by tracking distinct nucleoli within each nucleus. A, time-lapse series of wild-type, Lmna<sup>−/−</sup>, Lmna<sup>LCO/LCO</sup>, and Lmnb1<sup>LAP/LAP</sup> fibroblasts. The images shown were acquired at 5 min, 4 h, and 8 h. Wild-type nuclei (Lmna<sup>−/−</sup>) (first row) were very stable over time and showed only minor deformations, whereas Lmna<sup>LCO/LCO</sup> (second row) and especially Lmna<sup>−/−</sup> (third row) cells underwent large nuclear deformations over time. Deformations of Lmnb1<sup>LAP/LAP</sup> nuclei (fourth row) were comparable with those of wild-type nuclei. Red crosses denote the actual centroid of each tracked nucleolus, whereas white crosses mark the theoretical positions of these nucleoli based on a least-square fit assuming linear affine transformations (see “Experimental Procedures”). Deviations between the white and red crosses indicate nuclear deformations. The average deviation between the actual nucleolus positions and the least-square fit at each time point are plotted to the right. B, box and whiskers graph of time-averaged nuclear deformations. The box extends from the 25th to the 75th percentile, with an additional line marking the median. The whiskers indicate the minimum and maximum values. Lmna<sup>−/−</sup> MEFs had increased nuclear deformations compared with cells from wild-type littermates (***, p < 0.0001). Lmna<sup>LCO/LCO</sup> cells had less severe but still significantly increased nuclear deformations compared with wild-type littermates (***, p < 0.0001). Differences in nuclear deformations between Lmnb1<sup>LAP/LAP</sup> and wild-type cells were not statistically significant. C, overview of time-averaged nuclear deformation results, with all wild-type cells pooled into one group (***, p < 0.001 versus wild-type cells; the difference between Lmnb1<sup>LAP/LAP</sup> and wild-type cells was not statistically significant).
FIGURE 4. Lamins A and C are the primary contributors to nuclear mechanics. MEFs plated on silicone membranes were subjected to biaxial strain. The extent of induced nuclear deformations was measured and is expressed as a ratio of nuclear strain to applied membrane strain (normalized nuclear strain). In the box and whiskers graph, the box extends from the 25th to the 75th percentile, with an additional line marking the median. Whiskers indicate the minimum and maximum normalized nuclear strains.

A, Lmna<sup>−/−</sup> MEFs had increased normalized nuclear strain compared with cells from wild-type littermates (Lmna<sup>+/+;</sup> and Lmna<sup>+/−</sup> cells with reduced levels of lamins A and C showed elevated nuclear deformations under strain (***, p < 0.001 for wild-type versus Lmna<sup>−/−</sup> and Lmna<sup>+/−</sup> cells). B, Lmna<sup>−/−</sup> LCO/LCO fibroblasts had increased normalized nuclear strain compared with wild-type cells (**, p < 0.01 for wild-type versus Lmna<sup>−/−</sup> cells). Increases in nuclear strain in Lmna<sup>−/−</sup> and Lmnb1<sup>−/−</sup> cells were not quite significant compared with those in wild-type cells when analyzed by analysis of variance. C, Lmnb1<sup>−/+</sup> cells had slightly lower normalized nuclear strain compared with cells from wild-type littermates (**, p < 0.05). D, shown is an overview of the nuclear strain experiments, with all wild-type cells (WT) pooled into one group (***, p < 0.001 for wild-type versus Lmna<sup>−/−</sup> and Lmnb1<sup>−/−</sup> cells; the difference between Lmnb1<sup>−/+</sup> and wild-type cells was not statistically significant). E, multiple regression analysis was performed to evaluate the influence of specific lamin subtype levels on nuclear stiffness, represented by normalized nuclear strain values from the nuclear strain experiments. The expression levels of specific lamin subtypes were quantified by Western analysis and normalized to actin levels and are expressed relative to those of wild-type controls. Regressions were based on the mean values from 16 different cell lines for which normalized nuclear strain and protein levels results were available (supplemental Table 1). Results for each regression are presented in Table 1.
fraction of Lmnb1−/+ nuclei with a reduced contour ratio (in the range of 0.6–0.7, reflecting cells with nuclear blebs) (Fig. 2D).

To obtain an independent and quantitative assessment of nuclear shape, we applied elliptic Fourier shape analysis to the digitized nuclear cross-sectional outlines (26). Nuclei with a round or ellipsoidal shape had a large EFC ratio, whereas nuclei with an irregular shape or protrusions yielded a lower EFC ratio. The EFC ratio in Lmna−/− MEFs was smaller than that in wild-type cells (p < 0.001); the EFC ratio was also low in LmnaLCO/LCO MEFs (p < 0.05), although not as low as that in Lmna−/− cells (Fig. 2E). This trend was particularly evident in the frequency distribution of the EFC values (Fig. 2F, left and center panels). The frequency distribution of EFC ratios for Lmnb1Δ/Δ MEFs revealed a peak at lower EFC values, corresponding to cells with nuclear blebs (Fig. 2F, right panel). As a consequence, the median EFC ratio for Lmnb1Δ/Δ MEFs was lower than that for wild-type controls (p < 0.001, n ≈ 1300 for each group). These data demonstrate that the loss of specific lamin subtypes differentially affects nuclear shape and morphology. Whereas the loss of A-type lamins causes global changes in nuclear shape, the loss of lamin B1 causes localized blebs, and the overall nuclear shape remains largely normal.

Cells Deficient in A-type Lamins Have Altered Nuclear Dynamics—To assess differences in nuclear shape dynamics in Lmna−/−, LmnaLCO/LCO, and Lmnb1Δ/Δ MEFs, we quantified nuclear motion and deformation with time-lapse video microscopy (Fig. 3A) (19). Lmna−/− MEFs had significantly increased nuclear deformations compared with wild-type littermates (Fig. 3B), confirming earlier findings (19). LmnaLCO/LCO cells displayed a similar trend, but the average nuclear deformations were smaller than those in Lmna−/− MEFs, even though a few fibroblasts had nuclear deformations comparable with those in Lmna−/− cells (Fig. 3, B and C). In contrast, nuclear deformations in Lmnb1Δ/Δ MEFs were no different from those in wild-type cells (Fig. 3, B and C).

Lamins A and C Are Primary Regulators of Nuclear Stiffness—The nuclear shape changes observed in the time-lapse experiments could be caused by intracellular forces exerted from the cytoskeleton to the nucleus or through dynamic remodeling of the nuclear envelope or intranuclear structures. To explore the role of specific lamins in nuclear stiffness and stability independent of long-term intranuclear and cytoskeletal changes, we subjected Lmna−/−, Lmna+/−, LmnaLCO/LCO, and Lmnb1Δ/Δ MEFs to uniform biaxial strain.

Lmna−/− MEFs had significantly greater normalized nuclear strain compared with wild-type cells (Fig. 4A), indicating reduced nuclear stiffness; Lmna+/− displayed an intermediate phenotype (Fig. 4A). LmnaLCO/LCO MEFs also exhibited increased normalized nuclear strain (Fig. 4B) but to a lesser extent compared with Lmna−/− cells. LmnaLCO/LCO MEFs express more lamin C than do wild-type cells (16), so we also analyzed LmnaΔCO−/− cells, which have lamin C expression levels similar to those in wild-type cells. The nuclear deformations in LmnaLCO−/− and LmnaLCO/LCO cells were virtually identical (Fig. 4B), indicating that abnormalities in nuclear stiffness in LmnaLCO/LCO MEFs are due to the loss of lamin A and not to increased amounts of lamin C. Lmnb1Δ/Δ MEFs did not exhibit any changes in nuclear stiffness (Fig. 4, C and D), strongly suggesting that lamins A and C (and not lamin B1) are the key regulators of nuclear stiffness. These results were further validated by multiple regression analysis of normalized nuclear strain as a function of the expression levels of lamins A, B1, B2, and C based on the mean values of 16 different cell lines (Table 1). We found that normalized nuclear strain was inversely correlated with the cellular levels of lamins A and C and had no relationship to the levels of lamins B1 and B2 (Fig. 4E).

Lamin-deficient Fibroblasts Have Normal Cytoskeletal Architecture—To explore if altered force transmission to the nucleus could be responsible for some of the differences in nuclear deformations, we examined the cytoskeletal organization of microtubules and actin microfilaments by immunofluorescence. Changes in perinuclear microtubule organization and the presence of actin filament fragments have previously been described in some Lmna−/− MEFs (21), but our analysis revealed that all cell lines examined had similar fractions of these abnormalities and that cytoskeletal architecture in LmnaLCO/LCO, Lmna−/−, and Lmnb1Δ/Δ MEFs was comparable with that in wild-type controls (supplemental Fig. 1). Expression of actin and tubulin was also not significantly different between the cell lines (data not shown). These data suggest that the observed differences in nuclear deformation are indeed reflective of altered nuclear structure and function.

Defective Nuclear Stiffness in Lmna−/− MEFs Can Be Rescued by Exogenous Expression of Prelamin A—Because Lmna−/− MEFs displayed the most severe nuclear shape and stiffness defects, we evaluated if these abnormalities could be rescued by exogenous expression of fluorescently labeled lamins. Transient transfection with prelamin A-RFP resulted in significantly improved nuclear stiffness, whereas transfection with GFP-lamin C or GFP-lamin B1 had no effect on nuclear stiffness (Fig. 5, A and B). Interestingly, transient transfection with prelamin A-RFP, GFP-lamin C, or GFP-lamin B1 did not reduce the fraction of irregularly shaped nuclei, and expression of prelamin A-RFP and GFP-lamin C actually increased the fraction of cells with nuclear blebs. Unlike prelamin A-RFP, GFP-lamin C localized poorly to the nuclear envelope in Lmna−/− MEFs and was often seen in aggregates at the nuclear envelope and in the nucleoplasm (sup-

| Variable          | Intercept   | Slope          | R²     | Significance |
|-------------------|-------------|----------------|--------|--------------|
| Lamin A           | 0.355 (0.255–0.456) | −0.199 (−0.333 to −0.065) | 0.4203 | p < 0.01     |
| Lamin C           | 0.420 (0.063–0.556) | −0.197 (−0.327 to −0.067) | 0.4297 | p < 0.01     |
| Lamin B1          | 0.210 (0.068–0.352) | 0.035 (−0.102 to 0.171) | 0.0208 | NS (p = 0.59) |
| Lamin B2          | 0.173 (0.163–0.330) | 0.059 (−0.061 to 0.179) | 0.0739 | NS (p = 0.31) |
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Mildly Impaired Nuclear Mechanics Do Not Affect Cell Viability under Strain—To determine whether the abnormal nuclear stability and stiffness in Lmna^-/-, Lmna^+/-, and Lmna_LCO/LCO MEFs are associated with decreased cell viability, we measured cell death and apoptosis in fibroblasts subjected to 24 h of cyclic biaxial strain (19, 20). Lmna^-/- fibroblasts (but not Lmna^+/- or Lmna_LCO/LCO cells) had more cell death compared with wild-type cells (Fig. 6, A and B). These findings were further supported by an apoptosis assay, which showed an increased fraction of apoptotic cells in Lmna^-/- MEFs subjected to strain, but no increase in apoptosis in Lmna^+/- or Lmna_LCO/LCO cells (Fig. 6, C and D). These data indicate that a complete loss of A-type lamins is associated with reduced cell viability in response to mechanical stress, whereas a partial loss of A-type lamins is not.

Lmna^-/- Myotubes Have Impaired Nuclear Stiffness Similar to Lmna^-/- Fibroblasts—To explore if the nuclear defects observed in Lmna^-/- fibroblasts can also be found in other cell types that have different genomic organizations and cytoskeletal arrangements, we subjected Lmna^-/- skeletal myoblasts induced to differentiate into multinucleated myotubes to uniform biaxial strain and measured induced nuclear deformations (Fig. 7). Lmna^-/- myotubes had induced normalized nuclear strain values comparable with those seen in Lmna^-/- fibroblasts and significantly larger than those in myotubes from wild-type littermates. These results suggest that the measurements in MEFs reflect general defects in nuclear structure and could contribute to the tissue-specific defects found in muscular laminopathies.

DISCUSSION

We sought to better define the roles of lamins in nuclear shape, stiffness, and stability and in cell viability in response to strain. To approach this goal, we assessed these parameters in fibroblasts from a host of genetically modified mice (Lmna^-/-, Lmna^+/-, Lmna_LCO/LCO, Lmna_LCO/-, and Lmnb1^Delta). We identified striking abnormalities in nuclear mechanics in Lmna^-/- cells, with reduced nuclear stiffness and increased nuclear shape variability over time. In contrast, Lmna^+/- and Lmna_LCO/LCO MEFs had only relatively minor abnormalities in nuclear stiffness. Lmna^-/- cells also exhibited an increased susceptibility to cell death with the application of nuclear strain (19, 20), whereas Lmna^+/- and Lmna_LCO/LCO cells did not. Quite surprisingly, Lmnb1^Delta MEFs had normal nuclear stiffness and stability even though a significant fraction of the nuclei from those cells contained blebs.

The finding that Lmnb1^Delta MEFs had increased nuclear blebbing but normal nuclear mechanical properties suggests that the loss of lamin B1 might cause local disturbances in nuclear envelope structure without causing generalized defects in nuclear organization, stiffness, and shape stability. Interestingly, the quantity of lamins A and C, two proteins important in nuclear stiffness, appeared to be specifically reduced in Lmnb1^Delta nuclei at sites of nuclear blebbing but otherwise appeared to be normal (18). It is possible, of course, that the B-type lamins play a key role in regulating global nuclear stiffness, but that lamins B1 and B2 have largely redundant roles,

FIGURE 5. Transfection with prelamin A-RFP rescues nuclear stiffness but not nuclear shape abnormalities in Lmna^-/- fibroblasts. Lmna^-/- fibroblasts were transfected with prelamin A-RFP, GFP-lamin C, or GFP-lamin B1 and subsequently analyzed for changes in nuclear stiffness and shape compared with RFP/GFP-negative cells in the same dishes. A, normalized nuclear strain results. Cells were plated on silicone membranes and subjected to uniform biaxial strain (~5%) at 3–4 days post-transfection. In the box and whiskers graph, the box extends from the 25th to the 75th percentile, with an additional line marking the median. Whiskers indicate the minimum and maximum normalized nuclear strains. Prelamin A-RFP-positive cells had significantly decreased normalized nuclear strain (*, p < 0.05 versus RFP/GFP-negative cells), whereas nuclear deformations in all other cells were comparable with those in non-transfected Lmna^-/- cells (compare with Fig. 4). B, percentage of cells exceeding the 95th percentile (0.436) for normalized nuclear strain values for wild-type cells. Prelamin A-RFP-transfected Lmna^-/- cells had a significantly lower fraction of nuclei exceeding this threshold compared with Lmna^-/- MEFs transfected with GFP-lamin B1 or with GFP-lamin C or GFP/RFP-negative cells in the same dishes (**, p < 0.01). C, nuclear shape analysis. Lmna^-/- MEFs transiently expressing prelamin A-RFP, GFP-lamin C, or GFP-lamin B1 were stained with Hoechst 33342 nuclear stain 2–3 days after transfection, and nuclear morphology was evaluated by observers blinded for transfection status. Transient transfection with either lamin subtype did not significantly change the fraction of irregularly shaped nuclei but increased the fraction of cells with nuclear blebs (*, p < 0.05 versus RFP/GFP-negative cells; **, p < 0.01 versus RFP/GFP-negative cells).
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FIGURE 6. Lmna<sup>−/−</sup> but not Lmna<sup>+/+</sup> or Lmna<sup>LCO/LCO</sup> fibroblasts are more sensitive to mechanical strain. MEFs were subjected to 24 h of cyclic biaxial strain (1 Hz, 10% strain). Subsequently, strain-induced necrosis was measured by propidium iodide uptake, and the fraction of apoptotic cells was measured by DNA content analysis. A, Lmna<sup>−/−</sup> but not Lmna<sup>+/+</sup> MEFs had an increased number of propidium iodide (PI)-positive cells compared with wild-type cells and unstrained controls (***, p < 0.001 for strained Lmna<sup>−/−</sup> versus strained Lmna<sup>+/+</sup> and Lmna<sup>LCO/LCO</sup> cells; differences between unstrained cells were not statistically significant). B, the differences between Lmna<sup>LCO/LCO</sup> and wild-type cells were not statistically significant for strained or unstrained cells. C, Lmna<sup>−/−</sup> fibroblasts had increased fractions of apoptotic cells compared with wild-type cells, whereas Lmna<sup>+/+</sup> cells appeared to be normal (***, p < 0.001 for strained Lmna<sup>−/−</sup> versus strained Lmna<sup>+/+</sup> and Lmna<sup>LCO/LCO</sup> cells; differences between unstrained cells were not statistically significant). D, Lmna<sup>LCO/LCO</sup> fibroblasts did not have increased levels of apoptotic cells after strain application.

and that the impact of lamin B1 deficiency is masked by normal expression of lamin B2. We look forward to the availability of lamin B2 knock-out cells and lamin B1/B2 double knock-out cells so that the roles of B-type lamins in nuclear mechanics can be studied in more depth.

The present experiments extend our previous studies on Lmna<sup>−/−</sup> fibroblasts (19, 20) and provide new insights into the relative in vivo importance of lamins A and C. The fact that lamin C can largely compensate for a complete absence of lamin A is somewhat surprising, given earlier studies showing that lamin A plays a key role in directing the assembly of lamin C into the nuclear lamina (28, 32, 33). Recently, Motzsch et al. (34) reported that lamin C requires coexpression with GFP-lamin A to be targeted to the nuclear lamina. In the same series of experiments, Emery-Dreifuss muscular dystrophy mutations had different effects when expressed in the context of lamins A and C, suggesting distinct functional roles for the two A-type lamins (34). In our experiments, transient transfection of Lmna<sup>−/−</sup> MEFs with GFP-lamin C resulted in increased nuclear blebbing and aggregates at the nuclear envelope and in the nucleoplasm, and even Lmna<sup>−/+</sup> MEFs expressing high levels of GFP-lamin C had nuclear aggregates, whereas Lmna<sup>+/+</sup> cells expressing low levels of lamin C had normal incorporation of lamin C into the nuclear lamina (supplemental Fig. 2). These data suggest that lamin C incorporation is rate-limited and facilitated by lamin A. Exogenous overexpression of lamin C can easily overwhelm this system, particularly in Lmna<sup>−/−</sup> MEFs, but the correct incorporation of lamin C in Lmna<sup>LCO/LCO</sup> cells indicates that lamin A is not essential for correct lamin C incorporation. In contrast, prelamin A-RFP is correctly incorporated into the nuclear lamina (35) and rescues nuclear stiffness in Lmna<sup>−/−</sup> MEFs. Broers et al. (36) found that both GFP-labeled lamins A and C are stably incorporated into the nuclear lamina in wild-type cells, but that lamin C appears to be significantly more mobile within the nuclear interior, indicating the possibility of reduced polymerization into stable structures. On the basis of the latter findings, we suggest that lamin C might largely replace the structural function of lamin A at the nuclear lamina and is sufficient to recruit emerin to the nuclear envelope (supplemental Fig. 3); however, the increased mobility of lamin C in the nuclear interior of Lmna<sup>LCO/LCO</sup> fibroblasts could have led to somewhat altered nuclear shape dynamics in the time-lapse experiments and decreased nuclear stiffness in the nuclear strain experiments.

The fact that Lmna<sup>LCO/LCO</sup> fibroblasts have relatively minor
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abnormalities in nuclear deformation and do not develop necrosis and apoptosis in response to nuclear strain, combined with the health and vitality of Lmna<sup>LCO/1</sup>LCO mice (16), could be relevant to the development of strategies to treat certain laminopathies, in particular Hutchinson-Gilford progeria syndrome. In Hutchinson-Gilford progeria syndrome, lamin C synthesis is normal, but a point mutation in exon 11 perturbs mRNA splicing and leads to the accumulation of a mutant form of prelamin A in cells. This mutant prelamin A is entirely responsible for aging-like disease phenotypes. Thus, strategies to fight this disease by reducing prelamin A synthesis, e.g. with antisense- or RNA interference reagents, make sense. The finding that lamin A and prelamin A appear to be dispensable implies that it might be possible to implement this type of strategy. Along these lines, Fong et al. (16) have recently shown that a prelamin A-specific antisense oligonucleotide ameliorates the nuclear shape abnormalities in cells from a mouse model of progeria.

The interaction of different laminas in vivo is still unclear, although the binding of laminas A and C to lamin B1 has been observed in blot overlays (37), column binding assays (38), and two-hybrid assays (5, 39). Using an in vitro assay, Schirmer and Gerace (50) found that homotypic and heterotypic interactions of lamin B2 are less resistant to chemical dissociation compared with interactions between the other lamin subtypes, whereas lamin A interactions appear to be most stable. Delbarre et al. (40) demonstrated recently that laminas A and B1 polymerize in vitro in distinct homopolymers that can further interact with each other. Thus, it is easy to imagine that these laminas form cell-to-cell variability seen in the nuclear strain experiments. The interaction of different laminas in vivo is still unclear, although the binding of laminas A and C to lamin B1 has been observed in blot overlays (37), column binding assays (38), and two-hybrid assays (5, 39). Using an in vitro assay, Schirmer and Gerace (50) found that homotypic and heterotypic interactions of lamin B2 are less resistant to chemical dissociation compared with interactions between the other lamin subtypes, whereas lamin A interactions appear to be most stable. Delbarre et al. (40) demonstrated recently that laminas A and B1 polymerize in vitro in distinct homopolymers that can further interact with each other. Thus, it is easy to imagine that these laminas form distinct networks at the nuclear lamina and in the nucleoplasm that can differentially affect nuclear structure and function, and lamin B was implicated recently in mitotic spindle formation (41). Nonetheless, the finding that different lamin proteins can interact in vitro in heterotypic complexes implies that the loss of one lamin might have secondary effects on the levels of other laminas in cells. Indeed, there have been reports of altered levels of lamin B2 in the setting of certain lamin A/C mutations (42). In this study, we found no consistent abnormalities in the levels of laminas A and C in Lmna<sup>L1ΔC</sup> cells and no abnormalities in the expression of B-type laminas in Lmna<sup>−/−</sup> cells but increased levels of B-type laminas in Lmna<sup>L1/ΔC</sup>LCO cells. Cell-to-cell variability in the expression levels of specific lamin subtypes can contribute to the wide range of nuclear shape abnormalities within identical cell lines and could also be responsible for the large cell-to-cell variability seen in the nuclear strain experiments.

In summary, our experimental results suggest that laminas A and C are the primary contributors to nuclear stiffness. Lamin B1 does not significantly contribute to nuclear stiffness, although its absence clearly leads to some nuclear blebbing. Lmna<sup>−/−</sup> and Lmna<sup>L1/ΔC</sup>LCO cells have relatively minor abnormalities in nuclear shape and slightly reduced nuclear stiffness but no increase in susceptibility to cell death in response to strain. The latter findings are particularly relevant to progeria, as they suggest that antisense- or RNA interference-based therapies to reduce lamin A synthesis may have limited or no toxicity.
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