Absence of progeria-like disease phenotypes in knock-in mice expressing a non-farnesylated version of progerin

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Hutchinson–Gilford progeria syndrome (HGPS) is caused by a mutant prelamin A, progerin, that terminates with a farnesylcysteine. HGPS knock-in mice (LmnaHG/+ ) develop severe progeria-like disease phenotypes. These phenotypes can be ameliorated with a protein farnesyltransferase inhibitor (FTI), suggesting that pro- gerin’s farnesyl lipid is important for disease pathogenesis and raising the possibility that FTIs could be useful for treating humans with HGPS. Subsequent studies showed that mice expressing non-farnesylated progerin (LmnaHG−/− mice, in which progerin’s carboxyl-terminal −CSIM motif was changed to −SSIM) also develop severe progeria, raising doubts about whether any treatment targeting protein prenylation would be particularly effective. We suspected that those doubts might be premature and hypothesized that the persistent disease in LmnaHG−/− mice could be an unanticipated consequence of the cysteine-to-serine substitution that was used to eliminate farnesylation. To test this hypothesis, we generated a second knock-in allele yielding non-farnesylated progerin (LmnaCSIMHG) in which the carboxyl-terminal −CSIM motif was changed to −CSM. We then compared disease phenotypes in mice harboring the LmnaHG or LmnaCSIMHG allele. As expected, LmnaHG−/− and LmnaHG+/− mice developed severe progeria-like disease phenotypes, including osteolytic lesions and rib fractures, osteoporosis, slow growth and reduced survival. In contrast, LmnaCSIMHG−/− and LmnaCSIMHG+/− mice exhibited no bone disease and displayed entirely normal body weights and survival. The frequencies of misshapen cell nuclei were lower in LmnaCSIMHG−/− and LmnaCSIMHG+/− fibroblasts. These studies show that the ability of non-farnesylated progerin to elicit dis- ease depends on the carboxyl-terminal mutation used to eliminate protein prenylation.

INTRODUCTION

Hutchinson–Gilford progeria syndrome (HGPS), the classic progeroid syndrome of children, is caused by a mutant form of prelamin A, generally called progerin, that terminates with an internal 50-amino acid deletion within the carboxyl-terminal region of the protein. The deletion leaves progerin’s carboxyl-terminal CaaX motif intact, so the protein undergoes the usual CaaX motif posttranslational modifications (farnesylation of the cysteine, release of the last three amino acids and methyl- lation of the farnesylcysteine) (1–7). Recently, we created mice harboring an HGPS knock-in allele (LmnaHG) and identified severe disease phenotypes resembling those in humans with HGPS (8,9). The disease phenotypes in LmnaHG+/− mice were ameliorated with a protein farnesyltrans- ferase inhibitor (FTI), suggesting that the protein prenylation is important for disease pathogenesis (9–11). To further explore this issue, we created a knock-in allele encoding a non-farnesylated version of progerin (LmnaCSIMHG, in which progerin’s carboxyl-terminal −CSIM motif was changed to −SSIM) (12). The single amino acid substitution abolishes protein prenylation and the two subsequent processing steps (release of the last three amino acids and carboxyl methyl- lation). We anticipated that mice harboring that allele

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Thus, it is conceivable that the toxicity of SSIM progerin could be due to the cysteine-to-serine substitution. Furthermore, the primacy of the protein prenylation in disease pathogenesis. Lmna progerin was toxic and invariably caused the demise of tissues. But on the other hand, the fact that non-farnesylated with HGPS, perhaps by lowering levels of progerin in hopes that FTI therapy might ameliorate disease in humans types of mice harboring the LMNA csmHG/csmHG mice were used to create the csmHG mouse model. Western blots revealed that progerin from the mutant allele along with lamins A and C was eliminated with an FTI (Fig. 1C). The disease in mice harboring a Lmna nHG allele would be free of disease, but to our surprise they developed all of the same disease phenotypes found in Lmna nHG/+ mice and invariably succumbed to the disease (12). The disease in Lmna nHG/+ mice tended to be milder than in Lmna nHG/+ mice, but that was likely due to lower steady-state levels of progerin within tissues (12). In any case, the persistence of severe disease in Lmna nHG/+ mice, where the progerin was non-prenylated, raised doubts about the primacy of the protein prenylation in disease pathogenesis.

The severe disease in mice harboring a Lmna nHG allele sounded mixed messages. On the one hand, the fact that the disease was somewhat milder in Lmna nHG/+ mice raised hopes that FTI therapy might ameliorate disease in humans with HGPS, perhaps by lowering levels of progerin in tissues. But on the other hand, the fact that non-farnesylated progerin was toxic and invariably caused the demise of Lmna nHG/+ mice sounded a pessimistic note, implying that ‘anti-preneylation’ drug therapy would never be particularly effective. However, the pessimistic view is subject to a caveat. The progerin produced by the Lmna nHG allele terminates with –SSIM, while non-prenylated progerin in FTI-treated Lmna HG/+ mice would terminate with –CSIM. Although the amino acid substitution is subtle (amounting to the replacement of a single sulfur atom with an oxygen), its potential importance cannot be dismissed. Single amino acid substitutions in prelamin A and lamin C cause a potpourri of substitutions in prelamin A and lamin C cause a potpourri of potential importance cannot be dismissed. Single amino acid substitutions in prelamin A and lamin C cause a potpourri of potential importance cannot be dismissed. Single amino acid substitutions in prelamin A and lamin C cause a potpourri of potential importance cannot be dismissed.

We are not aware of any experimental strategy that would allow one to directly test the toxicity of non-farnesylated CSIM progerin, simply because there is no way to eliminate protein prenylation in vivo without altering the last four amino acids of the protein (the residues that trigger protein prenylation). But fully understanding the properties of non-farnesylated progerin is important. Accordingly, we generated a second non-farnesylated progerin allele, one that terminates with –CSM rather than –SSIM. CSIM progerin lacks the isoquinoline within progerin’s CaaX motif and cannot be prenylated, but it retains a carboxyl-terminal cysteine (like the CSIM progerin that accumulates during FTI treatment) (9).

In the current study, we compared, side-by-side, the phenotypes of mice harboring the Lmna nHG or Lmna csmHG allele.

**RESULTS**

We used gene targeting to create a mutant Lmna allele, Lmna csmHG, that yields exclusively non-farnesylated progerin (Fig. 1A). The Lmna csmHG allele is designed to produce non-farnesylated progerin and is identical to the Lmna nHG allele produced earlier (8) except that the isoquinoline within the CaaX motif was deleted. Targeted embryonic stem (ES) cells, which were identified by Southern blotting (Fig. 1B), were used to create the Lmna csmHG mouse model. Western blots revealed that Lmna csmHG mice produced progerin (and no lamin A or lamin C); Lmna csmHG mice produced progerin from the mutant allele along with lamins A and C from the wild-type allele (Fig. 1C).

To verify that the progerin produced by Lmna csmHG mice was not farnesylated, we incubated Lmna HG/+ and Lmna csmHG/+ fibroblasts with 8-anilinogeraniol (AG), a farnesol analog (11,12,18–21). AG is incorporated into anilinogeranyl diporphosphate in cells and then used as a substrate by protein farnesyltransferase (FTase). Western blotting with an AG-specific antibody was used to detect farnesylated proteins. Farnesylated proteins were detected between lamin A and lamin C in all cell lines, corresponding to lamins B1 and B2, which have migration patterns similar to progerin (Fig. 2). When we examined proteins immunoprecipitated with a lamin A/C antibody, we observed farnesylated progerin in Lmna HG/+ cells but not in Lmna csmHG/+ or Lmna HG/+ cells (Fig. 2). The incorporation of AG into proteins was eliminated with an FTI (Fig. 2).

The phenotypes of the Lmna nHG and Lmna csmHG mice were quite different. Neither Lmna csmHG/+ nor Lmna csmHG/csmHG mice exhibited progeria-like disease phenotypes. Body weight curves, survival curves and adipose tissue stores were normal in male and female Lmna csmHG/+ and Lmna csmHG/csmHG mice, indistinguishable from those in wild-type littermate control mice (Fig. 3). In contrast, Lmna nHG/+ and Lmna nHG/nHG mice exhibited abnormal body weight curves, reduced survival and reduced adipose tissue stores (P < 0.0001) (Fig. 3A–D). Also, Lmna nHG/+ and Lmna nHG/nHG mice developed osteolytic lesions and spontaneous rib fractures, whereas Lmna csmHG/csmHG mice did not (Fig. 4A–D). Finally, bone density was reduced in Lmna nHG/+ mice but was normal in Lmna csmHG/csmHG mice (Fig. 4E). These differences could not be explained by an unintentional mutation in the knock-in alleles; progerin transcripts from Lmna nHG/+ and Lmna csmHG/+ mice were sequenced and were identical, except for the targeted nHG allele termi-nates with –CSIM, while non-prenylated progerin in FTI-treated Lmna HG/+ mice would terminate with –CSIM.

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Certain missense mutations in LMNA are known to cause cardiomyopathy with conduction system disease (22), but cardiomyopathy is not a general feature of HGPS. At 12 months of age, Lmna nHG/csmHG mice exhibited slightly reduced left ventricular posterior wall thickness (Fig. 5A) and ejection fraction (Fig. 5B), as judged by echocardiography, but these abnormalities were mild. No instances of sudden death were observed in our Lmna csmHG/csmHG colony, even in mice >1-year-old, and no histopathological abnormalities could be identified (data not shown).

Misshapen nuclei are common in human HGPS fibroblasts (1,2,23). To determine whether Lmna csmHG fibroblasts develop misshapen nuclei, we determined the frequencies of nuclear blebs and folds in early-passage primary fibroblasts from Lmna nHG/+ and Lmna csmHG/+ embryos (n = 4 cell lines/genotype; >2000 cells/genotype). Lmna csmHG/csmHG fibroblasts (but not Lmna csmHG/+ cells) had more nuclear blebs than Lmna HG/+ fibroblasts (Fig. 6). Lmna nHG/csmHG fibroblasts had more misshapen nuclei than Lmna csmHG/csmHG cells, primarily because of a higher frequency of nuclear folds (Fig. 6). We also examined nuclei in liver sections from Lmna HG/+ and Lmna csmHG/csmHG mice (Fig. 7). Nuclear blebs were rarely observed in Lmna csmHG/csmHG and Lmna csmHG/csmHG liver sections. Interestingly, most of the progerin in Lmna csmHG/csmHG and
Lmna<sup>nHG/aHG</sup> mice was located at the nuclear rim, indistinguishable from the location of lamin A in Lmna<sup>+/+</sup> mice (Fig. 7).

The milder disease phenotypes in Lmna<sup>nHG/+</sup> mice, compared with Lmna<sup>HG/+</sup> mice, were accompanied by lower steady-state levels of progerin, both in embryonic fibroblasts and tissues of adult mice (12). Given the complete absence of disease phenotypes in Lmna<sup>csmHG/+</sup> mice, we initially suspected that progerin levels in Lmna<sup>csmHG/+</sup> and Lmna<sup>nHG/+</sup> fibroblasts (<i>n</i> = 4 cell lines/genotype) were similar, as were progerin levels in tissues of Lmna<sup>csmHG/+</sup> and Lmna<sup>nHG/+</sup> mice (except in the liver, where progerin levels in Lmna<sup>csmHG/+</sup> mice were slightly higher than in Lmna<sup>nHG/+</sup> mice). Progerin levels in Lmna<sup>csmHG/+</sup> and Lmna<sup>nHG/+</sup> fibroblasts and tissues were lower than in Lmna<sup>HG/+</sup> fibroblasts and tissues (<i>P</i> < 0.0001) (Fig. 8).

**DISCUSSION**

In 2004, Fong et al. (24) suggested that the farnesylated form of prelamin A might be the principal culprit in the pathogenesis of lamin A/C-related progeroid syndromes. That concept quickly gained steam. Within a year, Yang et al. (8) created an HGPS knock-in model (Lmna<sup>HG/+</sup> mice) and reported that the frequency of misshapen cell nuclei in Lmna<sup>HG/+</sup> fibroblasts could be reduced with an FTI. Within months, multiple laboratories reported similar observations (25–27). Shortly thereafter, Fong et al. (28) and Yang et al. (9) reported that an FTI ameliorated disease phenotypes in Zmpste24<sup>−/−</sup> and Lmna<sup>HG/+</sup> mice (9,28). The encouraging results led to a clinical trial of FTI treatment in humans with HGPS (29,30).

However, some of the enthusiasm about FTI therapy was dampened by the discovery of severe progeria-like disease phenotypes in mice expressing non-farnesylated progerin (Lmna<sup>nHG/+</sup>). At face value, the findings in Lmna<sup>nHG/+</sup> mice implied that the real culprit in HGPS might be pregerin’s 50-amino acid deletion (rather than its lipid anchor) and that any benefits of ‘anti-protein prenylation therapy’ would be limited. However, those conclusions depend on a key premise—that the effects of SSIM progerin are similar to those of the non-prenylated CSIM progerin that accumulates when Lmna<sup>HG/+</sup> mice are treated with an FTI. In the current study, we further evaluated the effects of non-prenylated progerin. We did so by creating a new knock-in allele for non-farnesylated progerin, Lmna<sup>csmHG</sup>, encoding a progerin in which the isoleucine of the CaaX motif is deleted. Remarkably, Lmna<sup>csmHG/+</sup> and Lmna<sup>csmHG/csmHG</sup> mice were free of...
progeria-like disease phenotypes. In contrast, Lmna$_{nHG}^{+/-}$ and Lmna$^{nHG/-}$ mice developed severe disease (slow growth, osteolytic lesions in the bones, reduced bone density, reduced adipose tissue stores and reduced survival). Nuclear shape abnormalities were also milder in Lmna$^{csmHG/-}$ and Lmna$^{csmHG/csmHG}$ fibroblasts. The electrophoretic mobility of progerin, lamin B1 and lamin B2 (all farnesylated proteins) are virtually identical; hence, the AG-specific antibody detects farnesylated proteins in all cell lines. However, when the A-type lamins (lamin A, lamin C and progerin) were immunoprecipitated, only progerin in Lmna$^{csmHG/-}$ cells was detected with the AG antibody.

Figure 2. Assessing the farnesylation of progerin in Lmna$^{csmHG/-}$ cells. Metabolic labeling experiments with a farnesol analogue, AG, to assess farnesylation of progerin. Lmna$^{+/-}$, Lmna$^{HG+/-}$ and Lmna$^{csmHG+/-}$ mouse embryonic fibroblasts were incubated with AG (30 μM) in the presence (+) or absence (−) of an FTI (ABT-100, 5 μM) for 2 days. Western blots of whole cell extracts were performed with an AG-specific antibody (green) and a lamin A/C-specific antibody (red). (A) Comparison of Lmna$^{HG+/-}$ and Lmna$^{csmHG+/-}$ mouse embryonic fibroblasts. (B) Comparison of Lmna$^{+/-}$ and Lmna$^{HG+/-}$ mouse embryonic fibroblasts. The electrophoretic mobility of progerin, lamin B1 and lamin B2 (all farnesylated proteins) are virtually identical; hence, the AG-specific antibody detects farnesylated proteins in all cell lines. However, when the A-type lamins (lamin A, lamin C and progerin) were immunoprecipitated, only progerin in Lmna$^{csmHG/-}$ cells was detected with the AG antibody.

We considered the possibility that progerin produced from the Lmna$_{csmHG}$ or Lmna$_{nHG}$ allele contained some unintended mutation that explained the different phenotypes, but this was not the case. Except for the targeted mutation, the sequences of progerin transcripts from Lmna$_{nHG+}$ and Lmna$_{csmHG+}$ mice were identical. Also, one could not ascribe the different phenotypes to different steady-state levels of progerin. The progerin levels in Lmna$_{nHG+}$ and Lmna$_{csmHG+}$ fibroblasts were similar, as were progerin levels in tissues of Lmna$_{nHG+}$ and Lmna$_{csmHG+}$ mice (the progerin levels in liver actually appeared to be somewhat higher in Lmna$_{csmHG+}$ mice).

In the description of the Lmna$_{nHG+}$ mice, Yang et al. (12) raised the formal possibility that the cysteine-to-serine substitution could be responsible for the toxicity of SSIM progerin. Given the subtle nature of the amino acid substitution, that possibility initially seemed unlikely. But the absence of progeria-like disease phenotypes in Lmna$_{csmHG+}$ and Lmna$_{csmHG/csmHG}$ mice means that this possibility cannot be dismissed. Why the CSM progerin did not yield progeria-like disease phenotypes is unknown, but it is conceivable that the free cysteine in the carboxyl-terminus of CSM progerin somehow protects from disease. If so, the CSM progerin that accumulates in the setting of FTI treatment might be non-toxic. At this point, we cannot state with confidence which of the two ‘non-farnesylated knock-in alleles’ yields the most faithful and relevant model for HGPS treatment, but we tend to think that it is the Lmna$_{csmHG}$ allele. In large part, we say this because the health and vitality of the Lmna$_{csmHG/csmHG}$ mice were so striking, but one cannot exclude the possibility that progerin’s 50-amino acid deletion is intrinsically toxic and that the isoleucine deletion in the Lmna$_{csmHG}$ allele neutralizes the toxicity. Also, the absence of progeria-like phenotypes in mice expressing CSM progerin is consistent with observations on farnesylated and non-farnesylated versions of full-length prelamin A. A farnesylated full-length prelamin A (in the setting of ZMPSTE24 deficiency) causes a severe progeroid syndrome in both humans and mice (6,24,31–38). In contrast, mice expressing non-farnesylated full-length prelamin A had no progeria-like disease phenotypes (although they eventually developed cardiomyopathy and sudden death) (21). In the current study, we found mild left ventricular dysfunction in Lmna$_{csmHG/csmHG}$ mice, but we never observed sudden death.

As noted earlier, the ideal experiment would be to create ‘non-farnesylated progerin mice’ without introducing any mutation into progerin’s CaaX motif. In theory, one could achieve this goal by eliminating the expression of the protein prenyltransferases, but this approach would be fraught with difficulties—simply because deficiencies of those enzymes elicit severe disease. For example, a deficiency of FTase in skin keratinocytes causes severe alopecia (39), and a deficiency of FTase in hepatocytes causes severe hepato cellular disease (Shao H. Yang, unpublished data). Moreover, it is possible that eliminating protein farnesylation would not be sufficient to eliminate the prenylation of progerin, as recent studies have suggested that progerin could be geranylgeranylated when FTase activity is inhibited with an FTI (40). Eliminating both protein farnesylation and protein geranylgeranylation in the liver is associated with more severe pathology than eliminating protein farnesylation alone (Shao H. Yang, unpublished data).

It is interesting that the progerin in the liver of Lmna$_{csmHG/csmHG}$ and Lmna$_{nHG/csmHG}$ mice was positioned quite normally at the nuclear rim. We did not find this particularly surprising. Although protein prenylation is often assumed to be crucial for the targeting of lamin A to the nuclear rim (8,23), recent observations indicate that this is not the case (21,39,41). For example, in mice that express mature lamin A directly (completely bypassing prelamin A synthesis and prelamin A farnesylation), the mature lamin A in mouse tissues reaches the nuclear rim quite normally (41). Also, farnesylated prelamin A is normally positioned at the nuclear rim in the tissues of knock-in mice expressing only non-farnesylated prelamin A (21). Finally, the prelamin A that accumulates in FTase-deficient skin keratinocytes is targeted to the nuclear rim (39).

In summary, mice harboring the Lmna$_{csmHG}$ allele are free of progeria. Even the homozygous mice, which produce exclusively progerin and no lamin A or lamin C, lack all of the hallmarks of progeria. The absence of progeria in Lmna$_{csmHG+}$ and Lmna$_{csmHG/csmHG}$ mice sounds an
encouraging note to those interested in treating progeria with drugs that inhibit protein prenylation.

MATERIALS AND METHODS

Generation of Lmna<sup>cmHG</sup> mice

To generate a mutant Lmna allele yielding non-farnesylated progerin (Lmna<sup>cmHG</sup>), we used a gene-targeting vector identical to one used to create a mutant allele yielding farnesylated progerin (Lmna<sup>HG</sup>) (8), except that it contained a 3 bp deletion in exon 12 (within the 5′-arm of the vector). The short deletion eliminates the isoleucine of the CaaX motif. The mutation was introduced into the 5′-arm of the vector with the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA) with primer 5′-CAGACAGGAGGTGGCATGT-3′ and a complementary reverse primer. The integrity of the gene-targeting vector was verified by restriction endonuclease digestion and DNA sequencing.

ES cell clones (strain 129/OlaHsd) carrying the Lmna<sup>cmHG</sup> allele were identified by Southern blotting with EcoRI-cleaved genomic DNA and a 348 bp 5′-flanking probe (8). The probe detects a 10.4 kb band in the wild-type Lmna allele and a 9.3 kb band in the Lmna<sup>cmHG</sup> allele. Targeted ES cells were used to generate male chimeric mice, which were bred with C57BL/6 females to generate heterozygous knock-in mice (Lmna<sup>cmHG/+</sup>). Lmna<sup>cmHG/+</sup> mice were intercrossed to generate homozygous mice (Lmna<sup>cmHG/cmHG</sup>). Mice were genotyped by PCR with genomic DNA and oligonucleotide primers 5′-TGAGTTACACCTGGCTGCAC-3′ and 5′-CAGACAGGAGGTGGCATGT-3′. The wild-type allele yields a 582 bp PCR product, while the mutant allele yields a 183 bp product.

The phenotypes of Lmna<sup>cmHG/+</sup> and Lmna<sup>cmHG/cmHG</sup> mice were compared, side-by-side, with Lmna<sup>HG/+</sup> and Lmna<sup>HG/HG</sup> mice (12). Lmna<sup>cmHG/+</sup> and Lmna<sup>HG/+</sup> mice were bred from chimeras; thus, they were genetically identical except for distinct CaaX motif mutations (with one C57BL/6 chromosome and one 129/OlaHsd chromosome). Homozygous knock-in mice had a mixed genetic background. All mice were weaned at 21 days of age, housed in a virus-free barrier facility with a 12 h light-dark cycle and fed a chow diet containing 4.5% fat.
Cell culture

Primary fibroblasts were isolated from E13.5 embryos and cultured in standard fibroblast culture medium (8,10,34,42). In some experiments, protein farnesylation was blocked by incubating cells for 48 h with a selective FTI (ABT-100, 5 μM).

Metabolic labeling studies to assess protein farnesylation

Fibroblasts were plated in six-well dishes and grown to 75% confluency. 8-Anilinogeraniol (AG, 30 μM in DMSO) (18,19) was added to the cell culture medium and incubated for 48 h in the presence or absence of ABT-100 (5 μM). The incorporation of AG into proteins such as progerin was detected by western blotting with an AG-specific monoclonal antibody (1:5000) (18). In some experiments, A-type lamins were immunoprecipitated with a goat anti-lamin A/C antibody (1:5000) (Santa Cruz Biotechnology). Protein samples were size-fractionated on 4–12% polyacrylamide Bis–Tris gels (Invitrogen, Carlsbad, CA, USA) and then transferred to nitrocellulose for western blotting. The antibody dilutions were 1:400 for goat anti-lamin A/C IgG (sc-6215, Santa Cruz Biotechnology), 1:5000 for anti-AG mouse IgG and 1:1000 for anti-actin goat IgG (sc-1616, Santa Cruz Biotechnology). After washing, the membranes were incubated with 1:5000 IRDye 800 anti-goat IgG (Rockland Immunochemicals, Figure 4. Bone disease in Lmna<sup>+</sup>/+, Lmna<sup>csmHG/cmshHG</sup>, Lmna<sup>nHG/+</sup> and Lmna<sup>nHG/nHG</sup> mice. (A–C) μCT scans of the thoracic spine in Lmna<sup>+</sup>/+, Lmna<sup>csmHG/cmshHG</sup> and Lmna<sup>nHG/nHG</sup> mice. Red arrowheads indicate rib fractures and surrounding callus. (D) Numbers of rib fractures in Lmna<sup>csmHG/+</sup> mice (n = 12 males, 12 females), Lmna<sup>csmHG/cmshHG</sup> mice (n = 9 males, 10 females), Lmna<sup>nHG/+</sup> mice (n = 9 males, 9 females) and Lmna<sup>nHG/nHG</sup> mice (n = 9 males, 8 females). (E) Bone density in the ribs of Lmna<sup>csmHG/cmshHG</sup>, Lmna<sup>nHG/cmshHG</sup> and Lmna<sup>nHG/+</sup> mice (n = 4 mice/genotype), as judged by μCT analyses. Error bars indicate SEM.

Protein extraction and western blots

Protein extracts were prepared from early-passage fibroblasts with a urea-containing solubilization buffer (43). To make protein extracts from mouse tissues, tissues (~150 mg) were snap-frozen in liquid nitrogen and then ground into a powder with a mortar and pestle. The powder was resuspended in 0.5 ml of urea solubilization buffer (43) and then homogenized with a glass tissue grinder for 2 min. The samples were then sonicated and cleared by centrifugation (14 000 g for 10 min). Extracts were size-fractionated on 4–12% polyacrylamide Bis–Tris gels (Invitrogen), and the separated proteins transferred to nitrocellulose for western blotting. Antibody dilutions were 1:400 for goat anti-lamin A/C IgG (sc-6215, Santa Cruz Biotechnology), 1:1000 for goat anti-actin IgG (sc-1616, Santa Cruz Biotechnology) and 1:5000 for IRDye 800 anti-goat IgG (Rockland Immunochemicals,
Primary fibroblasts were plated on coverslips in 24-well plates, and grown to 75% confluency. Fibroblasts were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and grown to 75% confluency. Fibroblasts were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and grown to 75% confluency.

**Immunofluorescence microscopy**

Primary fibroblasts were plated on coverslips in 24-well plates, and grown to 75% confluency. Fibroblasts were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and grown to 75% confluency. Fibroblasts were fixed in 3% paraformaldehyde, permeabilized with 0.2% Triton X-100 and grown to 75% confluency.

**Mouse phenotypes**

Body weights were assessed weekly for 48 weeks; numbers of surviving mice were also recorded weekly. **Lmna**+/-, **Lmna**cmHG/csmHG and **Lmna**cmHG/csmHG mice were euthanized at 12 months of age. **Lmna**HG/HG and **Lmna**HG/HG developed progressive inanition and were euthanized after their body weight dropped by 2 g within a week (as specified by UCLA veterinary staff). To assess the amount of adipose tissue in mice, the major fat pads (reproductive, inguinal and mesenteric) were removed and weighed. To assess bone disease, the thoracic cavity of **Lmna**HG/HG and **Lmna**HG/HG and **Lmna**+/- mice was photographed after removing the heart and lung, and the numbers of rib fractures were counted (12,24,28,45). To assess bone density, **Lmna**cmHG/csmHG, **Lmna**cmHG/csmHG, **Lmna**cmHG/csmHG and **Lmna**+/- mice were examined by X-ray micro-computed tomography (μCT) with the SkyScan 1172 system scanner (SKYSCAN, Belgium). Bone density of ribs was determined by μCT scanning in a cylindrical sample holder tube with a voltage of 55 kVp and current of 143 μA. Three-dimensional images were reconstructed in 2048 × 2048 pixel matrices, and a cortical volume of interest was defined for each measurement. The mineralized tissue was segmented with a threshold of 71 for all samples. A hydroxyapatite phantom of known density was used for calibration. To measure the left ventricular posterior wall thickness and ejection fraction, **Lmna**+/- and **Lmna**cmHG/csmHG mice were examined by echocardiography (21).

**Statistical analyses**

Body weight curves were compared with repeated-measures ANOVA and the log-rank test. The bone density, number of rib fractures and fat pad weights were compared with a two-tailed Student’s t-test. Differences in the percentages of misshapen nuclei were assessed with a χ²-test. The significance of survival differences was assessed with a Kaplan–Meier test.
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Conflict of Interest statement. None declared.

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