

Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging

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The premature aging disease Hutchinson–Gilford Progeria Syndrome (HGPS) is caused by a mutant lamin A (LA Δ 50). Nuclei in cells expressing LA Δ 50 are abnormally shaped and display a loss of heterochromatin. To determine the mechanisms responsible for the loss of heterochromatin, epigenetic marks regulating either facultative or constitutive heterochromatin were examined. In cells from a female HGPS patient, histone H3 trimethylated on lysine 27 (H3K27me3), a mark for facultative heterochromatin, is lost on the inactive X chromosome (Xi). The methyltransferase responsible for this mark, EZH2, is also down-regulated. These alterations are detectable before the changes in nuclear shape that are considered to be the pathological hallmarks of HGPS cells. The results also show a down-regulation of the pericentric constitutive heterochromatin mark, histone H3 trimethylated on lysine 9, and an altered association of this mark with heterochromatin protein 1 α (Hp1 α) and the CREST antigen. This loss of constitutive heterochromatin is accompanied by an up-regulation of pericentric satellite III repeat transcripts. In contrast to these decreases in histone H3 methylation states, there is an increase in the trimethylation of histone H4K20, an epigenetic mark for constitutive heterochromatin. Expression of LA Δ 50 in normal cells induces changes in histone methylation patterns similar to those seen in HGPS cells. The epigenetic changes described most likely represent molecular mechanisms responsible for the rapid progression of premature aging in HGPS patients.

histone methylation | heterochromatin | progeria

Hutchinson–Gilford Progeria Syndrome (HGPS) is a premature aging disease usually diagnosed in the first 12–18 months of life (1). HGPS is characterized by a rapid progression of disorders including hair loss, growth retardation, lack of s.c. fat, aged-looking skin, osteoporosis, and arteriosclerosis (2, 3). Patients with HGPS usually die from heart attacks or strokes at \approx 13 years (4). The common form of HGPS is caused by a conservative heterozygous mutation (1824 C>T) in the human nuclear lamin A (LA) gene (*LMNA*), which introduces a splice site resulting in the synthesis of LA with 50 amino acids deleted near its C terminus [mutant LA (LA Δ 50)] (5, 6).

Nuclear lamins are divided into A and B types. Lamins A and C (LA/C) are derived from *LMNA* by alternative splicing, whereas lamins B1 and B2 are derived from different genes. The B type lamins are expressed in every cell, whereas the expression of A type lamins is developmentally regulated. Lamins have a common tripartite structure with an α -helical central rod domain flanked by globular N- and C-terminal domains (7). The basic structural unit of lamins is a dimer consisting of two parallel and in-register protein chains that form a coiled coil through the association of their rod domains (8). Lamin dimers assemble in a head-to-tail fashion forming protofilaments that interact laterally to form numerous higher-order structures (9).

Lamins are the major components of the nuclear lamina, a protein network located adjacent to the inner nuclear membrane.

The lamina maintains the mechanical properties and shape of nuclei, and it has been proposed that it provides a molecular docking site for peripheral heterochromatin (7, 10, 11). Lamins are also distributed throughout the nucleoplasm, where they appear to be essential for DNA replication and RNA polymerase II transcription (7). Interest in the lamins has increased because of recent reports of \approx 200 mutations in *LMNA* causing >15 distinct diseases, collectively known as the “laminopathies” (12).

HGPS fibroblasts accumulate LA Δ 50 as a function of their age in culture and coincidentally display changes in nuclear shape and architecture, most notably a loss of heterochromatin (1). In this study, we examine changes in the epigenetic histone marks, H3K27me3 for facultative heterochromatin, histone H3 trimethylated on lysine 9 (H3K9me3), and H4 trimethylated on lysine 20 (H4K20me3) for constitutive heterochromatin (13), which take place in HGPS cells as they age in culture. The data define alterations in repressive histone lysine methylation (14) as early events in disease manifestation and suggest that HGPS-specific *LMNA* mutations induce perturbed epigenetic control of chromatin structure.

Results

To initiate these studies, we examined the inactive X chromosome (Xi) of female HGPS patient fibroblasts and controls. The Xi is identifiable as a heterochromatic domain usually associated with the nuclear lamina. Silencing of the Xi is regulated by trimethylation of lysine 27 in histone H3 (H3K27me3) and X-inactive specific transcript (*XIST*) RNA (15–17). Fibroblasts from an age-matched normal female sibling of an HGPS patient were used as controls. In early passages [passages 9–13 (p9–13)], \approx 94% of control cells ($n = 193$) contained an Xi closely associated with the lamina, as determined by immunofluorescence with anti-H3K27me3 (Fig. 1*A a–c* and *D*). This number decreased slightly to \approx 80% ($n = 103$) at later passages (p20–25) (Fig. 1*D*). In addition, punctate H3K27me3 staining was present throughout the nucleoplasm of control cells at all passages (Fig. 1*A a–c* and data not shown). In early-passage HGPS cells, \approx 57% ($n = 200$) of the cells possessed an Xi that reacted with anti-H3K27me3, which decreased to \approx 36% by p21 ($n = 186$; see Fig. 1*D*). Interestingly, \approx 58% ($n = 200$) of the H3K27me3-positive Xi consisted of loose arrays of discrete fluorescent

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Abbreviations: Xi, inactive X chromosome; H3K27me3, histone H3 trimethylated on lysine 27; H3K9me3, histone H3 trimethylated on lysine 9; H4K20me3, histone H4 trimethylated on lysine 20; HGPS, Hutchinson–Gilford Progeria Syndrome; LA/C, lamin A/C; LA Δ 50, mutant LA in HGPS cells; PFA, paraformaldehyde; sat III, satellite III; pn, passage n ; HEK293, human embryonic kidney 293.

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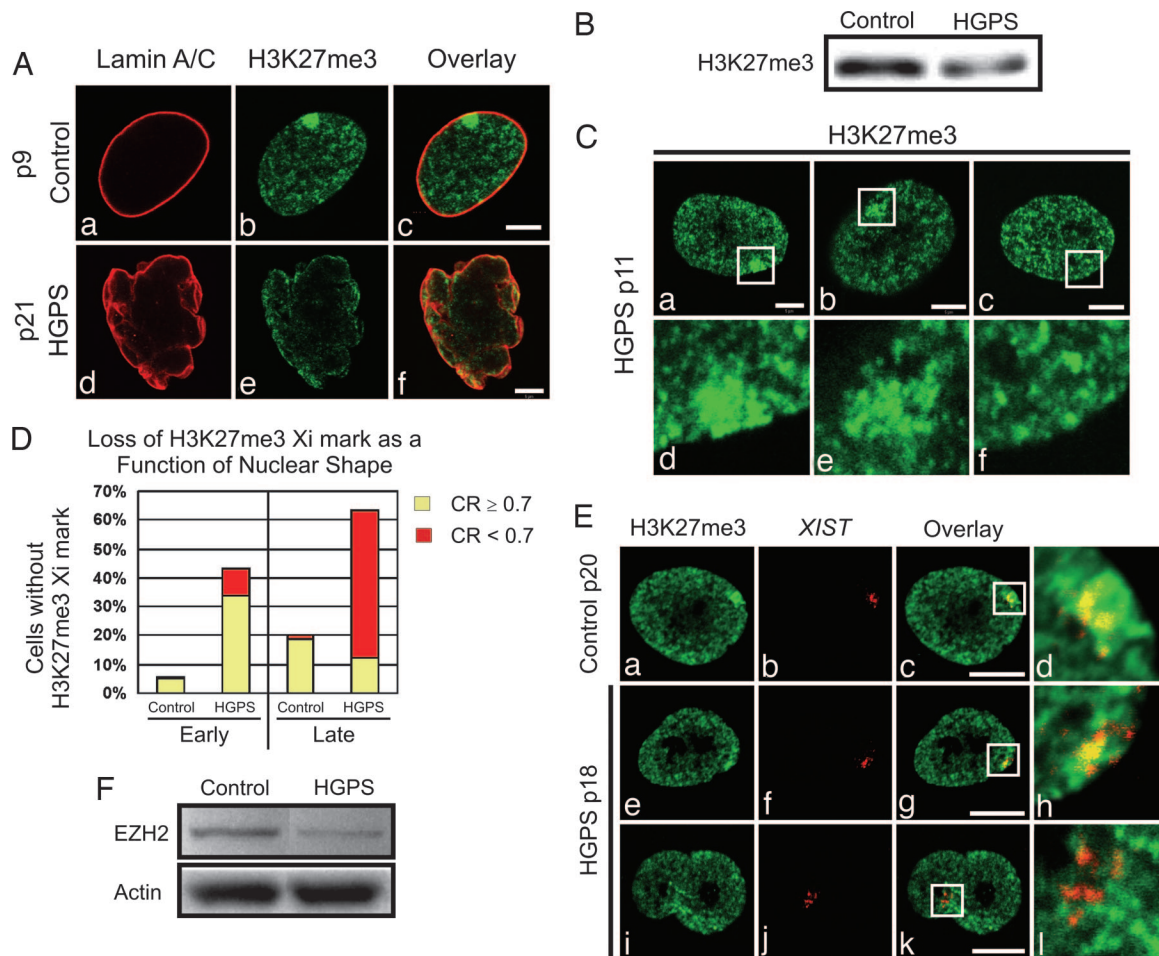


Fig. 1. H3K27me3 decreases and *XIST* RNA remains with Xi at all passages in HGPS cells. (A) Cells were processed for immunofluorescence with antibodies against LA/C (a and d) and H3K27me3 (b and e; overlay, c and f). Control cell nuclei at p9 displayed a distinct LA/C nuclear rim (a) associated with a compact Xi (b). The HGPS cells showed a change in nuclear morphology by p21 by using anti-LA/C (d), and there was no obvious Xi revealed by anti-H3K27me3 (e). (Scale bars, 5 μ m.) (B) There was a decrease in H3K27me3 by Western blot analysis of HGPS compared with control total cell extracts at p20. (C) In early-passage HGPS cells stained with anti-H3K27me3, the Xi appeared either as a uniformly stained compact domain (a and d) or a loose array of closely spaced granules (b and e), or it could not be detected (a typical lamina region in a nucleus with no Xi staining is depicted in the white box; c and f). d–f are enlargements ($\times 4$) of the regions in the white boxes in a–c. (Scale bars, 5 μ m.) (D) Nuclear contour ratios (CR; see also ref. 1) were determined for control and HGPS cells at early and late passages (nuclei with a CR \geq 0.7 were considered nonlobulated, and those with a CR $<$ 0.7 were considered lobulated). In early-passage HGPS cells, \approx 43% of the nuclei lost their H3K27me3 Xi mark, and \approx 80% of these were normally shaped. In late-passage cells, \approx 63% lost this mark, whereas \approx 20% of these nuclei were normally shaped. In early-passage controls, \approx 6% of the nuclei did not contain an obvious Xi, as distinguished by anti-H3K27me3. At late passages, this number increased to \approx 20%. (E) Control (a–d) and HGPS cells (e–l) were prepared for *XIST* RNA FISH and stained with anti-H3K27me3. (a–d) In controls at early and late passages (p13–20), *XIST* RNA was associated with the Xi, and \approx 93% ($n = 102$) also contained the H3K27me3 mark. (e–l) In HGPS cells, *XIST* FISH revealed an Xi at all passages, whereas by p17–18, the H3K27me3 staining on Xi was lost (\approx 53%; $n = 82$). (i–l) In lobulated HGPS nuclei lacking the H3K27me3 mark on Xi, the *XIST* RNA staining was more dispersed. d, h, and l are enlargements ($\times 5.6$) of the overlay regions in the white boxes (c, g, and k). (Scale bars, 10 μ m.) (F) Western blotting shows a decrease in EZH2 in HGPS cells compared with controls at p14. Actin was used as a loading control.

granules in p9–13 cells (Fig. 1C b and e). These loose arrays suggest the existence of transition states during the loss of the H3K27me3 mark (Fig. 1C a–f). Late-passage HGPS cells also showed a reduction in overall staining compared with controls, which was confirmed by immunoblotting (Fig. 1A d–f and B). Significantly, these observations revealed that many normally shaped early-passage HGPS cell nuclei lost the H3K27me3 mark on their Xi (Fig. 1C c and f and D). To quantify this, we correlated the H3K27me3 mark on Xi with the nuclear contour ratios of control and HGPS cells at different passages. In controls, the contour ratio remained 0.9 ± 0.2 from early to late passages, whereas in HGPS cells, there was a significant decrease from early (0.9 ± 0.2) to late (0.5 ± 0.2) passages. In early-passage HGPS cells, where the nuclear contour ratio was ≈ 0.9 , $\approx 34\%$ did not have a detectable Xi by H3K27me3 staining (Fig. 1D). These observations demonstrate that significant alterations

in chromatin regulation precede the nuclear shape changes that have been considered a pathological hallmark of HGPS cells (1, 18, 19).

We also determined whether there were changes in the association between *XIST* RNA and the Xi. All HGPS and control cells at early and late passages contained an Xi, as determined by *XIST* RNA FISH (Fig. 1E). When the Xi also stained with anti-H3K27me3, the signals colocalized (Fig. 1E d and h). When the Xi did not stain with anti-H3K27me3, the FISH signal was more dispersed (Fig. 1E i–l).

We also examined the expression level of EZH2, the methyltransferase responsible for the trimethylation of H3K27 (20). Immunoblotting demonstrated a significant reduction in EZH2 in HGPS cells compared with controls at p14 (Fig. 1F). There was also a reduction in the level of the mRNA encoding *EZH2* in HGPS relative to control cells, as determined by RT-PCR (see Supporting

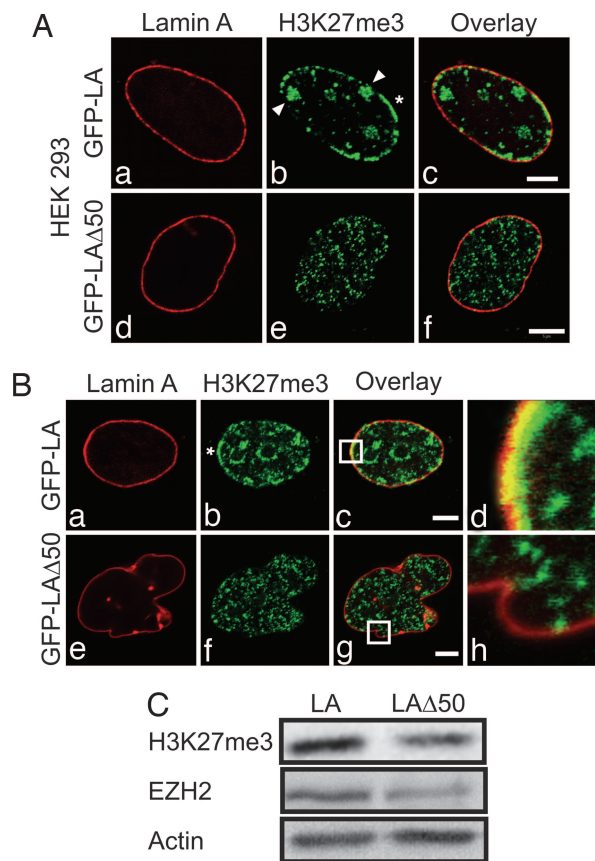


Fig. 2. Expression of GFP-LAΔ50 in HEK293 and HeLa cells. (A) HEK293 cells transiently expressing either GFP-LA or GFP-LAΔ50 were stained with anti-H3K27me3. In controls (GFP-LA), the lamina appeared normal (a), and H3K27me3 staining revealed several Xi (arrowheads, b) and arrays of peripheral heterochromatin (*, b) colocalizing with the lamina (c). Most cells expressing GFP-LAΔ50 showed a dramatic loss of Xi and peripheral heterochromatin staining with anti-H3K27me3, even in those retaining normal nuclear shapes (d–f). (B) HeLa cells expressing either GFP-LA (a–d) or GFP-LAΔ50 (e–h) were stained with anti-H3K27me3. GFP-LA-expressing cells displayed a normally shaped nucleus (a) and punctate H3K27me3 staining throughout the nucleus, with prominent arrays at the nuclear periphery (*, b), where it overlaps with the lamina (c and d). Expression of GFP-LAΔ50 caused lobulations in the nuclear envelopes of the majority of HeLa cells (e), and there was an overall loss of H3K27me3 staining, especially in the region of the lamina (f–h). Enlargements (×7.4) of the overlay areas indicated by white boxes (c and g) are shown (d and h). (C) Whole-cell extracts derived from HeLa cells expressing GFP-LA or GFP-LAΔ50 for 24–48 h were analyzed by immunoblotting. Note the reductions in H3K27me3 and EZH2 in cells expressing the mutant protein. Actin was used as a loading control. (Scale bars, 5 μm.)

Text and Fig. 6, which are published as supporting information on the PNAS web site). The *EZH2* mRNA level was decreased 10-fold in p25 HGPS cells relative to p14 control cells (see Fig. 6). In agreement with the FISH data, RT-PCR revealed no differences in the amounts of *XIST* RNA between early- and late-passage HGPS cells and late-passage controls (see Fig. 6 and data not shown).

We next studied the effects of transient expression of LAΔ50 in human embryonic kidney 293 (HEK293) cells. In the nuclei of nontransfected or GFP-LA-expressing cells, ≈80% ($n = 100$) contained multiple Xi that stained with anti-H3K27me3. Prominent staining not associated with the Xis was also observed in the lamina region and in a few nucleoplasmic foci (Fig. 2A a–c). In contrast, ≈20% ($n = 100$) of the cells expressing GFP-LAΔ50 contained a distinct Xi with a significant reduction in H3K27me3 staining in the lamina region (Fig. 2A d–f). This H3K27me3 staining was restricted to small foci distributed throughout the

nucleoplasm (Fig. 2A d–f). Furthermore, in all untransfected and transfected HEK293 cells examined, Xis were observed by *XIST* RNA FISH (data not shown).

The reduction in H3K27me3-positive chromatin domains related to the expression of LAΔ50 was further investigated by using HeLa cell lines induced to express GFP-LA or GFP-LAΔ50. In noninduced control and GFP-LA-expressing cells, small and large foci were present throughout the nucleoplasm, and there was a significant colocalization between LA and chromatin regions marked by H3K27me3 at the lamina (Fig. 2B a–d). Most nuclei of cells expressing GFP-LAΔ50 were convoluted, and there was an overall reduction in H3K27me3 fluorescence in ≈83% of the cells ($n = 100$; Fig. 2Bf). In addition, the large nucleoplasmic foci were not detected, and there was a substantial reduction of H3K27me3 staining in the lamina region (Fig. 2B e–h). Immunoblotting revealed a reduction in both H3K27me3 and EZH2 in cells expressing LAΔ50 (Fig. 2C).

The above data indicate there are major aberrations in the facultative heterochromatin of HGPS cells, as detected by anti-H3K27me3. To extend our analyses, we investigated possible changes in constitutive heterochromatin by examining H3K9me3, a known mark for pericentric heterochromatin (21). In early- and late-passage control cells, the staining pattern consisted mainly of small foci interspersed with a few larger foci both in the nucleoplasm and in the lamina region (Fig. 3A a–d and data not shown). Identical patterns were seen in early-passage HGPS cells (data not shown). In late-passage HGPS cells, there was an overall reduction of H3K9me3-positive foci in highly lobulated nuclei and a dramatic reduction in the lamina region (Fig. 3A e–h). An overall decrease of H3K9me3 in late-passage HGPS cells was also observed by immunoblotting (Fig. 3B).

We further investigated the relationship between constitutive heterochromatin and the expression of LAΔ50 by analyzing the patterns of H3K9me3 in the HeLa cell line expressing GFP-LAΔ50 (Fig. 3C d–f). Nuclei in control cells expressing GFP-LA show H3K9me3 foci distributed throughout the nucleoplasm and in linear arrays in the lamina region (Fig. 3C a–c). Expression of GFP-LAΔ50 resulted in a decrease in the number of nucleoplasmic and lamina-associated foci, especially in highly lobulated nuclei (Fig. 3C d–f).

Based on the changes in H3K9me3, we examined one of its binding partners, heterochromatin protein 1α (Hp1α; see ref. 22). In control nuclei, immunofluorescence observations revealed many bright foci containing both Hp1α and H3K9me3 (Fig. 3D a–d). In midpassage HGPS cells (p16), in addition to an overall reduction in fluorescence intensity for both Hp1α and H3K9me3, their association decreased. This loss of association appeared in many nuclei before extensive nuclear lobulation (Fig. 3D e–h). We also examined the relationship between H3K9me3 and kinetochores using CREST autoimmune serum (23). In controls, foci stained with H3K9me3 are closely associated with kinetochores (Fig. 3E a–d), whereas in midpassage HGPS cells, the number of such associations was reduced (Fig. 3E e–h). However, the overall H3K9me3 pattern in HGPS cells did not appear significantly altered at this passage number compared with p22 (compare Fig. 3A b and f with E b and f). In addition, transcript levels for SUV39H1 and SUV39H2 (24), the histone methyltransferases generating H3K9me3, were reduced in late-passage HGPS and control cells by RT-PCR (data not shown).

The loss of H3K9me3 associated with pericentric heterochromatin in HGPS cells suggested there may be an up-regulation of the transcriptional activity of pericentric regions. This possibility was investigated by examining the expression levels of two major pericentric DNA sequences, satellite III (sat III) and α satellite repeats (25, 26). There was no change in α satellite transcripts in HGPS cells even in late passages, as determined by RT-PCR (data not shown). In contrast, a significant increase in the level of chromosome 9 sat III transcripts was detected in mid- to late-

(100 mM NaCl/300 mM sucrose/3 mM MgCl₂/10 mM Pipes, pH 6.8) for 30 sec, washed in cytobuffer with 0.5% Triton X-100 for 5 min, washed in cytobuffer for 30 sec, and fixed for 10 min in 4% PFA in PBS. The cells were dehydrated in 70%, 80%, 95%, and 100% ethanol; air-dried; and hybridized overnight at 37°C with *XIST* RNA probes in hybridization buffer [2× sodium saline citrate (SSC)/0.3 M NaCl/30 mM Na-citrate (pH 7.0)/50% formamide]. Slides were washed three times for 5 min in hybridization buffer at 40°C, 10 min in 2× SSC at 40°C, and three times for 5 min in 4× SSC at 22°C.

sat III RNA. Cells grown on coverslips were prepared essentially as described (34). In some cases, cells were heat-shocked for 1 h at 42°C followed by recovery at 37°C for 3 h. Cells were washed in PBS (all steps were at 22°C unless otherwise specified), fixed in 4% PFA for 10 min, washed in PBS/100 mM Tris (pH 7.4), washed twice on ice in PBS/0.5% Triton X-100, washed on ice in PBS, dehydrated stepwise with ice-cold ethanol (70%, 80%, 90%, and 100%) for 5 min each, and then air-dried. The sat III probes (25) were hybridized overnight at 37°C. The cells were washed three times for 5 min in hybridization buffer, three times for 5 min in 2× SSC and for 15 min in 4× SSC/0.1% Tween-20; incubated for 60 min in 20 μg/ml FITC-avidin (Vector Laboratories) in PBS; washed three times for 3 min in PBST, then in PBS; incubated for 60 min in 10 μg/ml biotinylated anti-avidin D (Vector Laboratories) in PBS; washed three times for 3 min in PBST; and then washed for 3 min in PBS. After a 60-min incubation in 20 μg/ml FITC-avidin (Vector Laboratories) in PBS and two 3-min washes in PBST, then PBS, the cells were blocked for 30 min in PBST with 10% FCS. The cells were stained with anti-LA/C (Jol2; Chemicon; 1:200) for 60 min, washed twice for 3 min in PBST and for 3 min in PBS, then incubated for 60 min with goat anti-mouse IgG-Alexa 633 (1:300) (Molecular Probes) and washed in PBS. Cells were examined by confocal microscopy.

Preparation of FISH Probes. The DNA probes for *XIST* RNA FISH were amplified by PCR from a genomic bacterial artificial chromosome clone containing the *XIST* locus using the primer pairs 1aF (accctgtttttgttgacag)/1aR (ctcccaagtgtcgggatta), 1bF (aagacaattgcctggaatc)/1bR (gcacataacagccaagaaaa), and

12F (ctctcagacccttttgcag)/12R (ggccttgtctgtcaacatt). *XIST* RNA FISH probes were Cy3-labeled (see ref. 17), and hybridization was carried out as described (16). The probes used for sat III have been described (25). FISH probes were provided by Integrated DNA Technologies (Coralville, IA).

Expression of GFP-LA and GFP-LAΔ50. Transfections were performed by electroporation with a GenePulser Xcell (Bio-Rad) at 200 V and 950 μF using $\approx 1 \times 10^7$ cells and 10–20 μg of DNA in 400 μl of DMEM in a 4-mm gap cuvette using either pEGFP-myc-*LMNA* or pEGFP-myc-*hLMNAΔ150* (1). The cells were plated onto coverslips and processed for immunofluorescence after 24–48 h.

Stable HeLa Tet-On cell lines were prepared expressing either EGFP-myc-LA or EGFP-myc-LAΔ50. The coding regions from pEGFP-myc-*hLMNA* or -*hLMNAΔ150* were cut out with NheI and XbaI and ligated into the pTRE vector (Clontech) cut with XbaI. The resulting plasmids (pTRE-EGFP-myc-*hLMNA* and -*hLMNAΔ150*) were dual-transfected into HeLa Tet-On cells with the selection vector pTKHyg (Clontech) at a 20:1 ratio. The transfected cells were plated into two 10-cm culture dishes, incubated for 48 h, released by trypsinization, and each distributed to 10 10-cm dishes maintained in medium containing 200 μg/ml each G418 and hygromycin until visible colonies emerged. Single colonies were transferred to individual wells of 24-well plates. After the expansion of each colony, cells were induced with 2 mg/ml doxycycline for 24 h, and GFP fusion protein expression was analyzed by fluorescence microscopy.

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