Nesprin-2 giant safeguards nuclear envelope architecture in LMNA S143F progeria cells

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The S143F lamin A/C point mutation causes a phenotype combining features of myopathy and progeria. We demonstrate here that patient dermal fibroblast cells have dysmorphic nuclei containing numerous blebs and lobulations, which progressively accumulate as cells age in culture. The lamin A/C organization is altered, showing intranuclear and nuclear envelope (NE) aggregates and presenting often a honeycomb appearance. Immunofluorescence microscopy showed that nesprin-2 C-terminal isoforms and LAP2α were recovered in the cytoplasm, whereas LAP2β and emerin were unevenly localized along the NE. In addition, the intranuclear organization of acetylated histones, histone H1 and the active form of RNA polymerase II were markedly different in patient cells. A subpopulation of mutant cells, however, expressing the 800 kDa nesprin-2 giant isoform, did not show an overt nuclear phenotype. Ectopic expression of p.S143F lamin A in fibroblasts recapitulates the patient cell phenotype, whereas no effects were observed in p.S143F LMNA keratinocytes, which highly express nesprin-2 giant. Overexpression of the mutant lamin A protein had a more severe impact on the NE of nesprin-2 giant deficient fibroblasts when compared with wild-type. In summary, our results suggest that the p.S143F lamin A mutation affects NE architecture and composition, chromatin organization, gene expression and transcription. Furthermore, our findings implicate a direct involvement of the nesprins in laminopathies and propose nesprin-2 giant as a structural reinforcer at the NE.

INTRODUCTION

Lamins are type V intermediate filaments (IFs) and form the principle structural constituents of the nuclear lamina, a fibrous scaffold underlying the eukaryotic inner nuclear membrane. The nuclear lamina determines nuclear integrity and organization, nuclear pore positioning and constitutes a structural platform, which interconnects the genetic material with the nuclear membrane (1–3). The later interactions further expand the cellular functions of lamins, which include also chromatin organization, DNA replication, transcription and cell cycle regulation (4). Similar to cytoplasmic IFs, lamins are composed of a central α-helical coiled-coil (rod) domain, flanked by globular head and tail domains. The basic lamin unit is a /C24 50 nm two-stranded parallel coiled-coil homodimer. Head to tail associations of these rodlet-like units and staggered antiparallel lateral interactions of the rod domains ultimately yield a higher-order filamentous meshwork (5,6).

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Mammals contain three lamin genes, the ubiquitously expressed lamin B1 (LMNB1) and lamin B2 (LMNB2) genes and the developmentally regulated lamin A/C gene (LMNA). Lamins are classified as A-type (encoded by LMNA) or B-type (encoded by LMNB1 and LMNB2) based on their primary sequence and their biological properties (5). B-type lamins are considered as the fundamental nuclear envelope (NE) constituents, essential for cell viability and proper embryonic development (7). In contrast, A-type lamins have more specialized functions in terminally differentiated (primarily mesenchymal) cells, fulfilling essential functions in organ and tissue homeostasis. A-type lamins include lamin A, lamin C, lamin AΔ10 and the testis-specific lamin C2, all alternative spliced isoforms of a single LMNA gene (8–10). In contrast to lamin C (572 residues), the mature lamin A (646 residues) is produced by a series of complex post-translational processing steps of the prelamin A precursor. Prelamin A possesses a C-terminal CaaX-box motif undergoing farnesylation at its terminal cystein residue (11). Farnesylated prelamin A is the specific substrate for the mouse integral membrane metalloproteinase Zmpste24 (human FACE-1) which catalyses two distinct steps of endoproteolysis (12–14). First, the C-terminal aX residues are clipped off and the newly exposed last farnesyl-cysteine residue is carboxy-methylated by a membrane methyltransferase of the endoplasmic reticulum. During or after incorporation in the nuclear lamina, Zmpste24 cleaves the last 15 residues off and the mature lamin A is released.

Mouse and human genetic studies have elucidated the biological significance of A-type lamins. Lamin A/C knockout mice have a reduced lifespan, an impaired spermatogenesis and develop muscular dystrophy, peripheral neuropathy and cardiomyopathy (15–18). Unexpectedly, at least in the mouse, both prelamin A and lamin A appear to be dispensable, since lamin C-only mice did not display an overt phenotype (19).

In man, a plethora of different LMNA mutations cause a broad spectrum of more than 10 genetic diseases, collectively termed as laminopathies. (20–22). These include four premature ageing disorders, namely atypical Werner syndrome, mandibuloacral dysplasia, restrictive dermopathy and Hutchinson–Gilford progeria syndrome (HGPS) caused by different mutations in the LMNA gene (22).

HGPS is an extremely rare devastating segmental premature ageing disease (23). Children affected by HGPS appear normal at birth, but typically within a year develop growth retardation. Later symptoms include micrognathia, a beaked nose, loss of subcutaneous fat leading to extensive wrinkling of the skin, severe hair loss (alopecia), restrictive joint mobility, bone abnormalities, cardiovascular disease and progressive arteriosclerosis. The majority of known HGPS cases are caused by a single-nucleotide gain-of-function mutation in codon 608 (GGC > GGT; G608G), which activates a cryptic splice site deleting 150 nt of exon 11 during RNA splicing (24,25). The truncated LMNA mRNA transcript yields a deletion of 50 C-terminal amino acids from the prelamin A protein, known also as progerin (26). Progerin still retains the CaaX box motif but lacks the site for endoproteolytic cleavage and thus cannot be processed to mature lamin A. Progerin exerts a concentration-dependent dominant negative toxic effect, affecting the mechanical properties of the nuclear lamina in HGPS fibroblasts, and progressively leads to abnormalities in nuclear architecture and morphology (2,27).

The recently discovered lamin-associated nesprin-1 and nesprin-2 proteins are also implicated in NE organization and the physical integration of the nucleus with cytoskeletal filaments (28–30). Nesprins are multifunctional nucleo- and cytoplasmic spectrin-repeat-containing proteins, which associate with the NE by virtue of their highly conserved type II transmembrane domain (KASH domain) (31–33). Nesprin-2 is a highly complex gene coding for at least nine isoforms that vary enormously in their length and domain architecture (34,35). The largest transcript codes for a massive ~800 kDa scaffold protein (nesprin-2 giant/NUANCE) essential for emerin anchorage to the inner nuclear membrane in COS7 cells (28) and is composed of an N-terminal α-actinin type actin-binding domain (ABD), followed by a long spectrin-repeat-containing rod and a C-terminal KASH domain.

Although in recent years significant knowledge has been generated regarding the biochemical mechanism of action of progerin or farnesylated-prelamin A, very little is known about the pathophysiological effects of missense LMNA mutations causing progerias. A previous study reported a clinical case of a young girl with a new phenotype combining early-onset myopathy and progeria and identified the novel p.S143F LMNA point mutation as the underlying cause (36).

In the current study, we have characterized cultured dermal fibroblasts from this patient and have studied in addition the effects of ectopically expressed p.S143F LMNA GFP-fusion proteins in wild-type and nesprin-2 giant deficient fibroblasts. Our data show that the p.S143F mutation has devastating effects on the nuclear architecture and function. These degenerative effects are, however, counteracted by the concomitant expression of nesprin-2 giant, thus implicating nesprins directly in the pathogenic mechanism of the p.S143F LMNA mutation.

RESULTS

p.S143F LMNA4 fibroblasts display dramatic nuclear morphology changes

Serine-143 is an evolutionary highly conserved neutral residue within the unique 42 amino acid heptad repeat (coiled region 1B) specific to lamins (Fig. 1A and B). Figure 1A summarizes the currently known LMNA mutations causing progeria syndromes. Intrigued by the unique phenotype of the patient carrying the p.S143F LMNA mutation and the relative absence of cell biological data for progeroid mutations lying in the N-terminal half of lamin A/C, we have characterized cultured primary dermal fibroblasts obtained from a skin biopsy of the patient.

Since the classical hallmark of laminopathies, including HGPS cells, is the presence of nuclear architecture defects, we first examined the nuclear morphology of mutant fibroblasts by fluorescence microscopy. Labelling with the DNA-specific fluorescent dye Hoechst 33258 indicated that nuclear morphology was severely affected (data not shown). At passage 6, already 31.9% of mutant nuclei had an aberrant nuclear morphology.
Figure 1. Nuclear morphology defects in S143F LMNA cells. (A) Schematic representation of the names and positions of LMNA mutations identified in HGPS and atypical progeria syndrome (S143F is highlighted in light red colour). Functional domains of lamin A/C isoforms (bottom half) are denoted by colour; respective amino acid residue positions are indicated on the top. The LMNA exons, encoding for these domains, are indicated by an identical colour. (B) Coiled-coil lamin A/C residue S143 (denoted in yellow) is highly conserved in evolution. Amino acid sequence alignment of the rod segment (amino acids 120–170) of Homo sapiens lamin A/C (labelled Hs; GenBank accession no. AAH00511), Mus musculus lamin (Mm; GenBank accession no. P48678), Rattus norvegicus lamin (Rn; GenBank accession no. S27267), Xenopus laevis (Xl; GenBank accession no. NP_001039148), Sus scrofa lamin (Ss; GenBank accession no. CAJ28141) using the multalin computer program. Small amino acids are depicted in red, acidic ones in blue, basic in magenta; hydroxyl and amine in green and others in grey. Identical amino acids are indicated by asterisks. Heptad position assignment is shown below the alignment. (C) In contrast to control cells, (a) S143F LMNA fibroblasts (b and c) display an altered NE, as determined by indirect immunofluorescence using lamin A/C-specific antibodies. In mutant cells, lamin A/C displays often a honeycomb pattern (left cell in b) and is not evenly distributed along the NE, appearing as small nuclear aggregates (right cell in b) and as ruffles protruding into the cytoplasm (c). Nuclear shape defects vary in mutants from minor blebs to irregular-shaped and severely lobulated nuclei (left cell in b). Images shown were taken by confocal microscopy. Bars, 10 μm. (D) Electron microscopical examination of the nuclear morphology in S143F LMNA fibroblasts. In the control cells, a dense lamina is in contact with the inner face of the NE (a) and the nuclear pore complexes are visible at higher magnification (arrows in c). In the patient cells, blebs and invaginations of the NE are visible at low (arrows in b) and higher magnifications (large arrows in d), whereas the morphology of the nuclear pore complexes was not disturbed (small arrows in d). Bars, 1 μm. NPC, nuclear pore complexes; Cyt, cytoplasm; Nu, nucleoplasm.
morphology containing either minor NE blebs and/or prominent and multiple nuclear lobules. Similar to the nuclear defects observed in HPGS cells (2), the nuclear shape defects progressively accumulated with the number of cell divisions in culture. At passage 17, 56.4% of patient cells exhibited structural nuclear defects, in contrast to control human fibroblasts, where only 2.8% of the cells showed nuclear defects at even higher passages (Supplementary Material, Fig. S1A). These changes were not indicative of an arrest in proliferation or correlated to apoptosis. Cell cycle examination by fluorescence-activated cell sorter analysis did not indicate significant alterations between control and mutant fibroblasts (data not shown). It should be noted, however, that the proliferation of the mutant cells decreased significantly after passage 20, restraining culturing efforts beyond this stage.

To examine the subcellular localization and organization of A- and B-type lamins, we performed indirect immunofluorescence microscopy studies using specific antibodies against lamin A/C (Fig. 1C) or lamin B (Supplementary Material, Fig. S1C). Control cells revealed a prominent and uniform lamin A/C staining of the NE (Fig. 1Ca), whereas mutant cells exhibited a variable and aberrant lamin staining. Lamin A/C structures appeared as nucleoplasmic aggregates (Fig. 1Cb and c) and often displayed the previously reported honeycomb pattern (Fig. 1Cb) (37,38). In the majority of mutant cells, lamin A/C staining was not uniformly distributed along the NE, giving the nuclei a ‘rough’ appearance (Fig. 1Cc). Lamin B exhibited a uniform localization pattern (Supplementary Material, Fig. S1C); however, the staining appeared weaker in the deformed areas compared with the rest of the nucleus in mutant cells (Supplementary Material, Fig. S1Cb, arrow). Immunoblotting of whole cell extracts indicated slight reductions of lamin B expression levels, whereas lamin A/C was not significantly altered in patient versus control cells (Supplementary Material, Fig. S1B). Also, the lamin A/C ratio expression levels remained unaltered.

To examine more closely the ultrastructural changes of the mutant nuclei, we performed an electron microscopic analysis. In contrast to HPGS cells (2), the nuclear pores were distributed normally over the nuclear surface in early passage patient cells.

Abnormal localization of LEM-domain containing proteins in S143F LMNA patient cells

Integral inner nuclear membrane proteins require selective retention signals for their proper localization and function (40,41). The majority of those signals correspond to specific nucleoplasmic domains that interact directly with lamins and/or chromatin (20). Lamin mutations consequently affect directly their targeting mechanism and concomitantly the function and organization of INM structures. Therefore, we proceeded next with the examination of lamin-associated LEM-domain containing proteins such as emerin, LAP2α and LAP2β (42,43) in the mutant cells. LEM domains can associate with the barrier-to-autointegration factor (BAF), a conserved chromatin-binding protein (3). Emerin is an INM protein, which is found mutated or absent in the X-linked form of EDMD. LAP2α and LAP2β are two structurally and functionally different isoforms of the same gene (44). Immunofluorescence microscopy of mutant cells using emerin antibodies indicated a continuous staining at the NE, which occasionally contained abnormal emerin accumulations, giving an overall rough appearance to the mutant nuclei (Supplementary Material, Fig. S2Cg, arrows). Using an LAP2 anti-body, which recognizes all LAP2 isoforms, it was possible to observe that in contrast to wild-type cells (Fig. 2Aa), the intensity and the LAP2 localization pattern appeared weaker and heterogeneous in the mutant cells. Loss of the nuclear interior labelling, corresponding to LAP2α, as well as a weaker or discontinuous NE staining, corresponding to LAP2β was often evident (Fig. 2Ac, arrow, and d’). Immunofluorescence microscopy using LAP2α-specific antibodies confirmed the above subcellular localization abnormalities. In 99% of wild-type fibroblasts, LAP2α staining was confined to the nucleoplasm, whereas in 54% of the patient cells, LAP2α was by immunofluorescence not detectable (Fig. 2Ac, arrow) or found in the cytoplasm (Fig. 2Ad’). Immunoblot assays of whole cell extracts indicate a slight reduction in the Lap2α content and a 5-fold reduction in LAP2β protein levels in the mutant compared with wild-type cells (Fig. 2B), whereas emerin levels remain practically unchanged.

Nesprin-2 localization is affected in LMNA S143F dermal fibroblast cells

The localization of nesprin-1 and nesprin-2 at the NE is known to depend upon an intact lamin A/C network (28,35). This prompted us to investigate the distribution of these proteins in the mutant cells by immunofluorescence microscopy. Staining by a nesprin-1 C-terminal polyclonal antibody was confined to the NE in both wild-type and mutant cells where it largely co-localized with lamin A/C (Fig. 3Ad–i). Nesprin-2 C-terminal antibodies (pAb K1) indicated cytoplasmic (Fig. 3Bb, arrows) and NE immunofluorescence staining in wild type cells (Fig. 3Bb, arrow heads). In mutant cells with normal nuclear morphology, we consistently observed a clear nuclear rim staining for nesprin-2 (Fig. 3Be). In contrast,
in deformed mutant nuclei (Fig. 3Be, asterisks) nesprin-2 staining was mostly reduced or absent from the NE (Fig. 3Bh and i). A statistical evaluation of the nesprin localization in mutant versus wild-type cells indicated a slight increase in the percentage of mutant cells with a cytoplasmic nesprin-1 localization (Supplementary Material, Fig. S2A). These alterations are, however, far more dramatic for the nesprin-2 proteins. Whereas 8.4% of control cells display reduced or absent nesprin-2 NE staining, this percentage increased to up to 51.3% in the mutant fibroblasts (Supplementary Material, Fig. S2B). To further confirm our findings, we used emerin antibodies as an NE marker to verify whether its pattern correlated with an abnormal nesprin-2 localization. Indeed, mutant nuclei displaying a nesprin-2 mislocalization (Supplementary Material, Fig. S2Ce, asterisk, and h and i) also exhibited an altered emerin distribution.

Figure 2. Abnormal localizations for LAP2 and LAP2α in S143F LMNA patient cells. (A) Control cells were subjected to immunofluorescence using LAP2 (a′) and LAP2α (b′) antibodies. Note that all nuclei are stained. Patient cells were double-labelled with LAP2 (c′ and d′) and with LAP2α (c″ and d″) antibodies. LAP2 and LAP2α staining is less intense in some patient cells (arrow in c′ and c″, respectively). Note also the cytoplasmic localization of LAP2α (d″) and the uneven LAP2 NE staining in the mutant cells. A merged picture is shown in (c‴). The corresponding Hoechst fluorescence stainings (a–d) are shown. Bars, 20 μm. (B) Whole cell extracts of the indicated cell lines were subjected to SDS–PAGE followed by immunoblot analysis using LAPα,β and emerin-specific antibodies.
A normal emerin pattern (Supplementary Material, Fig. S2Cd, arrow head) was mostly accompanied by a normal confinement of nesprin-2 to the NE (Supplementary Material, Fig. S2Ce, arrow). In summary, the S143F LMNA mutation drastically affects nesprin-2 and mildly nesprin-1 localization.

Nesprin-2 giant expressing mutant fibroblast cells exhibit a normal nuclear morphology

To explore in more detail the nesprin-2 localization pattern in the LMNA mutant cells and to better understand its potential involvement in the pathogenesis of the S143F mutation, we performed co-immunolocalization studies employing the ABD directed nesprin-2 giant-specific mAb K20-478 (28,34) and the pAb K1 nesprin-2 C-terminal polyonal antibodies. Wild-type fibroblasts exhibit a heterogeneous nesprin-2 giant expressing cell population; in particular, proliferating cells (Ki67 positive) were consistently positive for nesprin-2 giant, whereas non-proliferating cells either lacked completely nesprin-2 giant or exhibited faint staining (data not shown). Similar to wild-type cells, only a subpopulation of mutant fibroblast cells was positive for the K20-478 antibody. Surprisingly, however, these mutant cells had a normal nuclear morphology and displayed a confined pAb K1 NE staining (Fig. 4A, arrows). Even mutant cells exhibiting only very faint nesprin-2 giant staining did not show nuclear abnormalities. Furthermore, nesprin-2 giant positive mutant nuclei had a smaller area and appeared more compact, compared with cells negative for K20-478 staining. In sharp contrast, mutant nuclei lacking nesprin-2 giant (Fig. 4A, asterisk) were consistently misshapen (Fig. 4A, arrow heads) and displayed a reduced pAb K1 staining at the NE. Our immunofluorescence observations were not restricted to low passage mutant cells and persisted even at higher passages (#17). A statistical evaluation of the samples indicated that at passage 11, 2.9% of control and 5.2% of mutant cells positive for nesprin-2 giant showed nuclear defects, whereas these numbers increased to 8.6 and 14.4% in control and mutant cells, respectively, at passage 17 (Fig. 4B). To determine more directly the molecular basis for these differences, we analysed the expression levels of nesprin-2 in control and mutant cell lysates. As shown in Figure 4C, in comparison to HaCaT keratinocytes, which strongly express nesprin-2 giant, a single faint 800 kDa band was detectable in the control, which was barely visible in the mutant even after prolonged ECL exposure. Immunoblotting with the pAb K1 antibodies verified that nesprin-2 giant levels are indeed decreased in the mutants when compared with controls. However, minor changes in the expression levels of the C-terminal isoforms migrating at \( \frac{1}{240} \) and \( \frac{1}{250} \) kDa (Fig. 4C, indicated by asterisks) were detected.

To document statistically the morphological differences between nesprin-2 giant positive or negative nuclei of mutant and control cells, we performed a morphometric analysis by calculating the average contour ratios (see Materials and Methods). As seen in Figure 4D, the contour ratios of nesprin-2 giant expressing control and mutant cells remain constant (>0.78) irrespective of the passage number. In addition, in control cells, the contour ratios appeared independent of nesprin-2 giant expression. However, mutant cells negative for nesprin-2 giant showed a wide range of lower contour ratios, decreasing from 0.72 in passage 11 to 0.61 in passage 15 (Fig. 4D and E). These data indicate a 1.44-fold increase in the nuclear perimeter of nesprin-2 giant negative nuclei compared with mutant nuclei expressing nesprin-2 giant. In summary, our findings suggest that nesprin-2 giant expression counteracts the harmful action of

Figure 3. The LMNA S143F mutation affects the nesprin-2 localization at the NE. Control and mutant cells were subjected to indirect immunofluorescence using lamin A/C (a, d and g), Nesprin-1 (Ab, e and h), and nesprin-2 C-terminal-specific polyclonal antibodies (pAb K1) (Bb, e and h). (A) Note the confined NE nesprin-1 staining in both, control (a–c) and patient cells (d–f). (g–i) Higher magnifications of the white dotted boxed areas indicating that nesprin-1 colocalizes with lamin A/C. (B) Control cells (a–c) show nesprin-2 staining both in the cytoplasm (b, arrows) and in the NE (b, arrowheads). Mutant cells (d–i) displaying nuclear deformations (e, denoted by asterisks) do not show nesprin-2 staining at the NE. (g–i) Higher magnifications of the white dotted boxed areas. In contrast to nesprin-1 (Ai), nesprin-2 is absent from the lamin A/C positive rim staining. Images shown were taken by confocal microscopy and merged to visualize co-localization. Bars, 10 \( \mu \)m.
the S143F *LMNA* mutation on the nuclear morphology and propose a structural reinforcer role for nesprin-2 giant at the nuclear membrane. Transient transfection experiments employing short 2.5 kb nesprin-2 fusions composed of an ABD and the KASH domain lacking the massive central spectrin-repeat-containing rod failed, however, to rescue the mutant defects (data not shown).

Figure 4. Mutants expressing nesprin-2 giant exhibit a normal nuclear morphology. (A) Cells were subjected to indirect immunofluorescence using the nesprin-2 polyclonal pAb K1 and nesprin-2 giant-specific K20-478 monoclonal antibodies. Mutants lacking a K20-478 staining (asterisk) have an abnormal nuclear shape (arrowheads), in sharp contrast to cells positive for K20-478 (indicated by arrows; see also insets). Insets are higher magnifications of the white dotted boxed areas. Bars, 10 μm. (B) Histogram representing a statistical evaluation of K20-478 positive cells (300 cells counted), which were assessed for nuclear deformations. Note the reduced number of nuclear abnormalities in mutant cells expressing the nesprin-2 giant isoform both in young and in aged cell cultures. (C) Western blotting analysis of equal amounts of HaCaT (control), control/mutant fibroblast cell extracts (see Coomassie blue-stained gel) using nesprin-2-specific antibodies indicates a slight reduction in the nesprin-2 giant (arrowhead) expression levels in the mutant. Levels of C-terminal nesprin-2 isoforms remain, however unaffected (asterisks). Note the higher expression levels of nesprin-2 giant in keratinocytes compared with fibroblasts. (D) Nuclear contour ratio analysis of *LMNA* S143F and control fibroblasts. Cells were subjected to indirect immunofluorescence using nesprin-2 giant antibodies and nuclei were stained with DAPI. In early and late passage control fibroblasts, no statistical difference for the nuclear contour ratio in K20-478 negative and K20-478 positive stained nuclei (*P* > 0.05) can be detected. Early and late passage mutants lacking a K20-478 staining have a significant decreased nuclear contour ratio when compared with cells expressing K20-478 (*P* < 0.001). (E) Relative frequency distribution of the contour ratio values presented in (D). Early and late passage wild-type nuclei display high contour ratios with a distinct peak of values. The early and late passage *LMNA* S143F cells having a K20-478 staining show a distinct peak and a contour ratio value distribution similar to the wild-type. In contrast, the early and late passage *LMNA* S143F cells lacking a K20-478 staining show a wider range of nuclear contour ratios with a shift towards the lower values. Note that in aged *LMNA* S143F cells (right graph) lacking a K20-478 staining, the severity of NE deformations increases.

*LMNA* S143F affects chromatin organization and transcription

It has been demonstrated that lamins and lamin-associated proteins bind to chromatin and that the disruption of nuclear lamin polymers inhibits RNA polymerase II-dependent transcription (45). In a previous study, we have shown that in
topology and gene expression are the histone proteins. The histones are subject to numerous covalent modifications that regulate gene expression (46). Therefore, we decided to analyse the localization of histone H1 and acetylated histones. Histone H1 is involved in chromatin condensation (46), whereas histone acetylation increases chromatin accessibility (47). In 42% of the patient cells, histone H1 concentrated at the poles of the nuclei (Supplementary Material, Fig. S3f, arrows) and in 53% of mutant nuclei histone acetylation was not detectable (Supplementary Material, Fig. S3, compare g' with h', arrow). The dramatic alteration of the distribution of phosphorylated RNA polymerase II, histone H1 and acetylated histones suggests an influence of LMNA S143F on the chromatin topology, organization and transcriptional activity. We did not observe any changes in the subcellular localization pattern for RNA polymerase I or for nucleolar proteins (data not shown).

The aberrant distribution of phosphorylated RNA polymerase II, nesprin-2 proteins and LAP2α in ~50% of the patient cells further stimulated us to investigate whether these alterations coincided within the same mutant cell. In double-labelling experiments, we analysed the localization of nesprin-2 and LAP2α with respect to phosphorylated RNA polymerase II. We could show that the reduction or aberrant localization of nesprin-2 and LAP2α correlates with the altered distribution of phosphorylated RNA polymerase II within the same cells (Fig. 5D and G, arrows, and E, F, H and I). Only in very few mutant cells with nuclear defects, the aberrant phosphorylated polymerase II distribution did not coincided with an altered nesprin-2 subcellular localization (Fig.5D and G, arrowheads). In summary, these results support the interpretation that the LMNA S143F mutation disorganizes the chromatin arrangement and influences gene expression by affecting chromatin accessibility and RNA polymerase II-dependent transcription.

Furthermore, most nesprin-2 giant expressing mutant cells not only had a normal nuclear morphology but also displayed a proper subcellular localization for various nuclear and NE proteins (data not shown).

Ectopic expression of the LMNA S143F mutation generates an aberrant nuclear phenotype similar to that observed in patient cells

To determine the LMNA S143F mutation as the primary cause of the observed cellular phenotype, we overexpressed wild-type (LMNA-GFP; Supplementary Material, Fig. S4) and mutated GFP-Lamin A fusion proteins (LMNA S143F–GFP; Fig. 6) in human wild-type fibroblasts. In contrast to LMNA-GFP expressing cells, which appeared normal, cells transfected with the mutant protein and examined by direct fluorescence microscopy exhibited the typical lobulated nuclei (Fig. 6Aa and b). Similar to the LMNA S143F patient cells, transmission electron microscopy revealed the existence of an abnormal NE and the presence of NE protrusions into the cytoplasm (Fig. 6Ac and d, arrows). The frequency of the nuclear phenotype alterations in transfected cells corresponded to that observed in patient fibroblast cells.

Furthermore, we analysed whether the distribution of various nuclear proteins was also affected in LMNA cells from an AD-EDMD patient with an LMNA R377H mutation, the distribution of the phosphorylated active RNA polymerase II isoform was dramatically altered (39). We found that the S143F lamin A mutation preferentially affects also the localization of the phosphorylated form of RNA polymerase II. In control cells, the immunofluorescence with RNA polymerase II antibodies revealed a normal, characteristic pattern of nuclear speckles distributed uniformly in the nuclei (Supplementary Material, Fig. S3a and c'). In patient cells, the distribution of the unphosphorylated, inactive form of RNA polymerase II was in 80% of mutant nuclei similar to the controls or concentrated abnormally at the poles of the nuclei but only in the remaining 20% of mutant cells (Supplementary Material, Fig. S3, compare b' with d'). However, the distribution of the phosphorylated RNA polymerase II was altered in 42% of patient cells and either appeared to concentrate at the poles of the nuclei (Supplementary Material, Fig. S3d', arrows) or to accumulate abnormally in the cytoplasm (Fig. 5D–F). Suitable markers to determine chromatin

Figure 5. Mislocalization of nuclear proteins in LMNA S143F cells. Double immunostaining analysis of patient cells by using antibodies against phosphorylated RNA pol II (D–F) and nesprin-2 (pAb K1; G and H) or LAP2α (I). The mislocalization RNA pol II and nesprin-2 (arrows in D, G and J; H and K) as well as RNA pol II and LAP2α (F, I and L) occurs in the same cells. Only few cells with a nuclear morphology defect show an aberrant localization of RNA pol II and a proper localization of nesprin-2 in the NE (arrowhead in D, G and J). Merged pictures are shown in (G–I). The corresponding Hoechst fluorescence stainings are shown in (A–C). Bars, 20 μm.
S143F–GFP-transfected human fibroblasts. We examined the distribution of phosphorylated RNA polymerase II and LAP2α (Fig. 6B) by immunofluorescence microscopy and found an abnormal localization of phosphorylated RNA polymerase II (data not shown) and LAP2α exclusively in transfected cells (Fig. 6Ba, arrow). In summary, these results identify the LMNA S143F mutation as the primary determinant in the genesis of the aberrant nuclear phenotype.

To further substantiate the potential role of nesprin-2 giant in the pathogenesis of the LMNA S143F mutation, we performed transfection studies with three different cell lines, which differed dramatically in the nesprin-2 giant expression levels. They are human HaCaT keratinocyte cells, which are high nesprin-2 giant expressers, human dermal wild-type primary fibroblasts expressing intermediate levels and the mouse C3H/10T1/2 cell line where nesprin-2 is expressed only in minor amounts (28) (Fig. 4C).
number of nuclear abnormalities is reciprocal to the nesprin-2 giant expression (Fig. 6C). The highest number for nuclear deformities was observed in mouse and then in human fibroblast cells. HaCaT cells, however, expressing LMNA S143F–GFP did not display any significant nuclear alterations. In contrast, the transfection with the mutant protein had immediate effects on the nuclear shape even after day 1 in mouse and human fibroblast cells. These defects were more dramatic at day 2 in both cell lines suggesting an accumulative harmful action of the S143F LMNA mutation (Fig. 6C). Our observations were not limited to the NE structural features but indicated that the mutant protein affects also NE protein distribution. Immunolocalization studies for LAP2 isoforms indicated that their localization was not affected by the presence of the mutant GFP fusion in HaCaT cells (Fig. 6D). Fibroblast cells, however, exhibited an aberrant non-uniform localization of these proteins along the NE (Fig. 6D, indicated by asterisks). These effects can be attributed directly to the S143F LMNA mutation, considering that ectopic expression of LMNA-GFP in the same cell lines did not cause alterations (Supplementary Material, Fig. S4).

Figure 7. Nesprin-2 giant counteracts the negative effects of the LMNA S143F mutation on the nuclear morphology. (A) Primary DFs from nesprin-2 giant knockout mice (b–b’ and d–d’) and wild-type mice (a–a’ and c–c’) were transiently transfected with LMNA S143F–GFP or LMNA-GFP control constructs and examined 3 days post-transfection by immunofluorescence microscopy using the nesprin-2 giant-specific mAb K56-386. Nuclei were stained with DAPI. Note that in primary wild-type fibroblasts nesprin-2 giant is present at the NE (a’ and c’, arrows), whereas knockout fibroblasts are negative for nesprin-2 giant staining (b’ and d’). Similar to non-transfected fibroblasts, nesprin-2 giant knockout fibroblasts transfected with the wild-type LMNA-GFP fusion (b–b’) did not exhibit a significant increase in nuclear deformations. Ectopic expression of the mutant lamin A (d–d’, asterisk), whereas the ectopic expression of the mutant LMNA-GFP fusion in wild-type primary fibroblasts leads to mild NE deformations (c, asterisk). Insets are higher magnifications of the white dotted boxed areas. Bars are 10 μm. (B) Histogram representing a statistical evaluation (300 cells counted for each experiment) of the nuclear deformities observed in wild-type and nesprin-2 giant knockout cells after transfection with wild-type and mutant LMNA-GFP fusions. Note the higher percentage of cells displaying LMNA S143F induced nuclear deformations in the absence of the nesprin-2 giant protein. (C) Nuclear contour ratio analysis of the cells investigated in (A and B). Note that the ectopically expressed LMNA S143F–GFP fusion decreases significantly the contour ratio of nesprin-2 giant deficient cells, whereas wild-type LMNA does not lead to significant changes in the knockout cells.
Altogether, our data demonstrate that the LMNA S143F mutation accounts for the altered nuclear phenotype both in patient and in transfected cells.

Ectopic expression of LMNA S143F–GFP in nesprin-2 giant knockout fibroblasts increases the amount and severity of nuclear deformations

To directly prove that the absence or reduction of nesprin-2 giant renders nuclei more susceptible to the deleterious action of the S143F LMNA mutation, we expressed wild-type and mutant LMNA-GFP fusions in nesprin-2 giant deficient cells. Primary dermal fibroblasts obtained from nesprin-2 giant knockout mice exhibit irregular-shaped nuclei (Fig. 7Ab; arrow). Twenty percent of the cell population shows nuclear morphology abnormalities and a significant decrease in their contour ratio (0.77) when compared with wild-type cells (0.89; Fig. 7B and C). Nesprin-2 giant localization in the primary dermal fibroblasts was examined using the monoclonal antibody K56-386, which is directed against the mouse nesprin-2 giant N-terminus. In control fibroblasts, an NE staining for nesprin-2 giant is detectable (Fig. 7Aa’ and c’; arrows), whereas it is absent in nesprin-2 giant knockout fibroblasts (Fig. 7Ab’ and d’).

Overexpression of the wild-type LMNA-GFP in nesprin-2 giant knockout fibroblasts does not lead to a significant induction (Fig. 7Ab and b’) or increase in nuclear deformations compared with non-transfected fibroblasts (Fig. 7B). In contrast, the ectopic expression of the LMNA S143F–GFP induces a considerable increase in the severity and the amount of nuclear deformations (Fig. 7Ad’, asterisk) in the knockout fibroblasts when compared with wild-type transfected cells (Fig. 7Ac, asterisk). Indeed, a statistical evaluation (300 cells counted) and a concomitant morphometric analysis indicate that the expression of the mutant LMNA increases the percentage of cells harbouring nuclear defects and leads to a significant decrease in the contour ratio from 0.77 (untransfected knockout cells) to 0.66 in transfected cells lacking nesprin-2 giant (Fig. 7C). In contrast, the contour ratio drops from 0.89 in untransfected wild-type cells to 0.84 in wild-type cells expressing the mutant LMNA protein (Fig. 7C).

**LMNA G608G and Zmpst24−/− mutants expressing nesprin-2 giant exhibit a normal nuclear morphology**

To explore whether nesprin-2 giant exhibits a general protective function on NE architecture, we extended our studies by examining human fibroblasts harbouring the ‘classical’ LMNA G608G mutation (24,25) and dermal fibroblast cells established from the Zmpst24−/− mice (12,13). Immunofluorescence examination of control and mutant cells (12,13) using nesprin-2 giant antibodies confirmed our LMNA S143F-based conclusions (Fig. 8A and B). Although the results were more pronounced in the LMNA G608G cells when compared with the Zmpst24−/− mutants, they unequivocally indicated that the presence of nesprin-2 giant is indicative of a normal nuclear morphology (Fig. 8A and B). The absence of nesprin-2 giant in the mutants was accompanied by severe nuclear morphology defects (Fig. 8A). The majority of human wild-type fibroblasts (94.2 ± 2.5%) expresses nesprin-2 giant, and only 2.9 ± 1.6% among them display NE defects. Although in the LMNA G608G mutants the number of nesprin-2 giant expressing cells decreases to 24.8 ± 7.1%, still only a minor amount of the nuclei (1.3 ± 1%) are abnormal. In the mutants, 75.2 ± 7.2% of cells are negative for nesprin-2 giant and the vast majority among them (73.7 ± 7.7%) displays an abnormal-shaped nucleus (Fig. 8B). Similar results were obtained in Zmpst24−/− mutants, only 13.6 ± 3.5% of nesprin-2 giant expressing mutants displayed nuclear deformations, which increased to 40.3 ± 3.5% in nesprin-2 giant negative cells (data not shown).

Interestingly, inhibition of farnesylation by using the FTI-277 compound in LMNA S143F and Zmpst24−/− mutants not only ameliorates the nuclear morphology defects...
of the mutants but also coincides with the restoration of the nesprin-2 localization at the NE (data not shown).

DISCUSSION

Over the last decade, a remarkable number of disease-specific mutations in the human LMNA gene have been identified. The underlying molecular mechanism, however, by which these mutations exert their harmful action, causing devastating tissue-specific diseases, is still poorly understood.

In an effort to better understand the pathology of the S143F lamin A/C mutation, which causes a unique phenotype, we examined the mutant dermal fibroblasts. We show that this mutation severely affects both nuclear architecture and function. Similar to other progeroid syndromes including Werner syndrome (48), atypical Werner syndrome (49) and the classical HGPS G608G silent mutation (2), S143F mutant cells exhibit also dramatic nuclear morphology defects, which progressively accumulate as cells age in culture.

Besides the unprecedented clinical features of the patient harbouring the S143F mutation (36), we observed NE ultrastructural changes in mutant cells, which to our knowledge have not been reported in other laminopathies yet. The nuclear membranes appear to detach locally from the underlying lamina, whereas in other areas, preserved lamina structures are clearly visible within these aberrant finger-like protrusions. These morphological abnormalities are not caused by a detachment of the outer and inner nuclear membranes. Thus, this ultrastructural phenotype is clearly distinct from the irregular ONM and INM spacing described for Sun1/2 knockdown cells (33). In fact, endogenous Sun1 was confined to the NE even in highly altered nuclei of mutant cells (data not shown), confirming therefore previous data from our laboratory (31) and others (33,50) that Sun1 does not rely upon A-type lamins for its proper NE localization.

Another mutation has been also reported at lamin A/C amino acid position S143. The substitution of Ser143 by proline (S143P) is associated with familial dilated cardiomyopathy, characterized by severe heart failure, progressive atrioventricular conduction defects and sudden death (51). Consistent with the data, which have been reported for other lamin mutations, different missense mutations at this sequence position give rise to different phenotypes (22).

The functional significance of this region in premature ageing is further underlined by the fact that three additional mutations in close proximity to S143 also cause progeroid syndromes. Heterozygous R133L and L140R mutations have been reported to cause atypical Werner syndrome (52), whereas the E145K mutation causes HGPS (25). The S143 residue does not reside within the coiled coil interface (position c in the heptad repeat amino acid sequence) (6,53) but rather occupies the surface of the coiled coil. Thus, the structural topology and particularly the substitution by a bulky, hydrophobic amino acid might not affect the lamin A/C dimer structure but rather perturb intermolecular interactions. In such a scenario, the S143F mutation would exert a dominant negative effect on lamin assembly and dynamics. This concept is in line with the abnormal organization of the lamin A/C network and the segregated distribution of lamin B in the mutant cells. Furthermore, fluorescence recovery after photobleaching studies indicate that S143F-LMNA-GFP fusions are less dynamic compared with the wild-type lamin A protein (our own unpublished data).

Additional proofs for a major defect in the nuclear lamina are the observed functional consequences on other nuclear proteins. The most dramatic nuclear alterations in mutant fibroblasts carrying the S143F mutation were the drastic reduction in LAP2β levels and the mislocalization of LAP2α and nesprin-2 C-terminal isoforms. Their altered distribution in the mutants is in agreement with their well-established tight associations with lamins. Lamina-associated polypeptide (LAP)2α is the non-membrane-bound LAP2 isoform that forms complexes with nucleoplasmic A-type lamins and chromatin (44), whereas LAP2β is an integral inner nuclear membrane protein binding to both lamin B and chromatin (41).

Mutant cells exhibit major changes in chromatin organization and topology. It is known that A-type lamins interact directly with DNA through their C-terminus (54), a domain that is not directly affected by the S143F mutation. An alternative explanation of the observed effects may be the alterations in the expression level of LAP2β and the mislocalization of LAP2α proteins, respectively. Both proteins share an identical LEM motif at their N-terminus essential for binding to BAF, a conserved DNA-binding protein implicated in chromatin organization (55). LAP2β is known as a transcriptional represor, which interacts with a class-I histone deacetylase (HDAC3) at the NE to induce histone H4 deacetylation (56). Thus, changes in LAP2β expression could have a direct impact on vital nuclear functions such as transcription. Transcription may be further impaired by the abnormal distribution of active RNA polymerase II in the mutant cells. This functional defect is also indicative of nuclear lamina alterations, since it is known that an altered nuclear lamina organization inhibits RNA polymerase II-dependent transcription (45). From our results, it is tempting to speculate that this LMNA mutation is indirectly involved in transcription regulation, by imposing a "higher order" of chromatin structure on genes which are in NE proximity or/and by modulating interactions with transcription factors involved in differentiation or in cell cycle progression such as the retinoblastoma protein (57,58). Indeed, pRB, a lamin A-binding protein, plays a role in muscle and fat cell differentiation as well as in cellular senescence (59,60), suggesting that a deregulated pRB activity could be associated to laminopathies.

The aberrant localization of the nesprin-1/-2 C-terminal isoforms in mutant cells is consistent with their well-documented dependency upon A-type lamins for proper nuclear targeting (28,35). The observed defects were, however, more severe for nesprin-2 than for nesprin-1 suggesting therefore, that although these molecules share similar modular organizations they may differ functionally, i.e. in their interaction partners and consequently in their molecular interplay with other nuclear elements. S143F is the first documented mutation in the LMNA gene shown to affect specifically nesprin-2. A homozygous Y259X LMNA mutation was reported to affect the subcellular distribution of nesprin-1α (61). Expression of nesprin-2 giant in the LMNA S143F mutant cells was
consistently associated with a normal nuclear morphology and function and appears to be a prerequisite for the localization of the C-terminal nesprin-2 isoforms. It is known that the ectopic expression of C-terminal nesprin segments harbouring the KASH domain is sufficient for NE targeting (28,31,34). Therefore, our findings suggest that nesprin-2 giant may stabilize the shorter C-terminal isoforms either by interaction with common binding partners or alternatively through homo-oligomerization of its spectrin repeats similar to the already described self-association of nesprin-1α (62). In support of this, nesprins-1 and -2 do not seem to interact with each other in vitro (35). Thus, the nesprin-1 isoforms present in mutant cells lacking nesprin-2 giant may not be able to exert a stabilizing effect on the localization of the shorter nesprin-2 variants.

Nesprin-2 giant appears to have a general role in rendering the NE less susceptible to mutant LMNA-induced nuclear deformations. In all three examined progeria cell lines, nesprin-2 giant presence at the NE was indicative of a normal nuclear shape. The mutated laminas we studied unmistakably affect negatively the nesprin-2 giant expression levels and the number of nesprin-2 giant expressing cells. Our hypothesis is that nesprin-2 giant and the investigated LMNA mutants exert opposing actions on each other and that the outcome of this interplay dictates the occurrence or the absence of an NE phenotype.

Taken together, our data clearly support a structural reinforcer role for nesprin-2 giant at the nuclear membrane. Such a function fits well to the already known role of spectrin-repeat-containing proteins to serve as structural platforms in the maintenance and organization of cytoplasmic membrane structures (63–65). Whether the absence of nesprin-2 giant at the NE is the primary cause for the ‘rough’ appearance of the LMNA S143F mutant nuclear membrane warrants further examination. Of particular interest is our finding that ectopically expressed LMNA S143F did not affect nuclear function and morphology in epithelial cells highly expressing nesprin-2 giant. Accordingly, if this is applicable at the tissue and organ level, one would logically assume minor defects in tissues/organs, which highly express nesprin-2 giant. In vivo, expression levels of nesprin-2 are high in the epidermis, kidney, liver, brain and in skeletal muscle (34) (our own unpublished data). The early-onset myopathy of the patient underlines that the pathology of our patient is, however, more complex. A plausible explanation for the existence of a phenotype in this particular tissue, which highly expresses nesprin-2 giant, would be that the skeletal muscle is more susceptible to lamina disturbances than the epidermal or neuronal cells, considering the enormous mechanical stress it undergoes. Furthermore, both nesprins-1 and -2 giant levels are elevated during muscle differentiation (35,66) (our own unpublished data); thus, the mutation may specifically affect myoblast cells, which are not protected by the presence of the nesprin-2 giant isoforms. As a consequence, the regeneration process of the skeletal muscle may be compromised. The latter is a hypothetical explanation, which is currently widely discussed to explain the pathology of some laminopathies (67,68). Further studies are needed to reveal and to entangle the spatiotemporal hierarchy of the various molecular events that are initiated by the presence of the mutated LMNA S143F protein.

Currently, two prevailing non-exclusive models exist addressing the deleterious action of lamin mutations namely, the ‘structural’ and the ‘gene expression’ model (67,69). The ‘structural’ model underlines the scaffolding properties of A-type laminas, proposing that mutant laminas affect nuclear structure and organization, thus rendering mutant cells more susceptible to mechanical stress. The ‘gene expression’ model is based on the fact that lamin associates with chromatin and chromatin-associated factors. The data we present here are supportive of the view that both the ‘structural’ and the ‘gene expression’ function of lamin A play a role in the pathogenesis of S143F LMNA mutant patient cells. More importantly, however, our data implicate nesprins in the pathogenic mechanism of the S143F mutation, proposing that nesprin proteins may be in general important molecular players in the pathogenesis of other laminopathies. Although the nesprin research remains a Sisyphus challenge still in its infancy, their examination in envelopopathies and healthy tissues may provide novel insights into key biological processes.

**MATERIALS AND METHODS**

**Patient and cells**

The young female patient G12660 with a phenotype of progeria carries a specific missense mutation in the LMNA gene (c.428 > t, p.S143F) encoding the proteins laminas A and C.

Human control fibroblast cells, G12660, LMNA G608G and Zmpste24−/− skin fibroblast cells were cultured in Minimum Essential Medium (Invitrogen, Karlsruhe, Germany) with 10% FCS, 1% penicillin/streptomycin and 7.5% bicarbonate. Cells were grown at 37°C and 5% CO₂ incubator.

For the FTI assays, LMNA S143F fibroblasts, mouse Zmpste24−/− fibroblasts and human wild-type dermal fibroblasts were treated with 20 µM FTI-277 (Calbiochem) for 42 h and then processed for further analysis.

**Antibodies**

Ascites fluids of the following primary monoclonal antibodies were used: X223 specific for the vertebrate B-type lamin B2 (70), R27 against human laminas A and C (71). Mouse monoclonal antibodies against emerin (NCL-emerin) were purchased from Medac (Hamburg, Germany), histone H1 from ICN Biomedicals (Berkshire, UK) and nucleoporins (mAB414) from Hiss Diagnostics (Freiburg, Germany); rabbit antibodies against acetylated lysine and histone H3 from Upstate (Dundee, UK); heterochromatin associated protein 1 goat antibodies from Santa Cruz Biotechnology (Heidelberg, Germany). Mouse monoclonal antibodies are directed against the phosphorylated CTD region of RNA polymerase II (RNA pol II) which is located within the C-terminal domain of the large subunit of RNA pol II [V/22 (39); H14 (72)], the unphosphorylated CTD region of RNA polymerase II (8WG16) (73), Lamin B Receptor (LBR, guinea pig) (74), RNA-polymersase I (S18, human) (75), LAP2 (13d4, mouse) (76), LAP2α (rabbit, a kind gift from Roland Foisner) (77), nesprin-2 giant (K20-478, mouse monoclonal) (34), nesprin-2 (pAb K1, rabbit) (28) and nesprin-1/Enaptin (rabbit) (28). Antibodies against digoxigenin (a-Dig, mouse)
for the TUNEL assay were obtained from Boehringer Mannheim GmbH, Germany.

**Gel electrophoresis and immunoblotting**

Proteins were resolved by 12% SDS–PAGE (78). For immunoblots, proteins were electrophoretically transferred to nitrocellulose (79). The membrane was blocked with 10% non-fat dry milk in TBST (10 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween-20) followed by antibody incubation at room temperature for 60 min with anti-emerin, Lap2β, Lap2α and nesprin-2. After several washes with TBST, the nitrocellulose was incubated with the secondary peroxidase-coupled anti-mouse antibodies (Dianova, Hamburg, Germany) at a dilution of 1:10 000 in TBST with 10% dry milk followed for 24 h at 4°C. The blots were washed again and bound antibodies were visualized using the enhanced chemiluminescence detection system (Luminol; p-Cumarsäure, Sigma-Aldrich, Steinheim, Germany). Nesprin-2 proteins were detected as described (34).

**Immunofluorescence microscopy**

Cultured cells grown on cover slips were fixed for 10 min in –20°C methanol, transferred to –20°C acetone for 1 min and air-dried. Alternatively, the cells were fixed with 2–4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized for 5 min with 0.1% Triton X-100 on ice and incubated immediately with primary antibodies.

Fixed cells were incubated with the primary antibodies for 30–60 min at room temperature. The antibodies were, respectively, diluted: anti-nesprin-1 and anti-nesprin-2 pAB K1 1:50 in PBS; R27, V/22; H14, 8WG16 1:100 in PBS; anti-emerin and X223 1:200 in PBS; anti-LBR, 13d4, Lap2α 1:500 in PBS; mAB414 1:5000 in PBS. After washing with PBS, the samples were incubated for another 30 min with the appropriate secondary antibody conjugated to Texas red, FITC, Cy2 and Cy3 (Dianova; diluted 1:50 in PBS). The samples were then counterstained with the DNA-specific fluorescent dye Hoechst 33258 (5 μg/ml), washed in PBS, air-dried from ethanol for 1 min and mounted in Mowiol (Hoechst, Frankfurt, Germany).

Photographs were taken with a Zeiss Axiophot equipped with epifluorescence optics and the appropriate filter sets (Carl Zeiss, Oberkochen, Germany) or with a confocal laser scanning microscope Leica TCS SP-2 AOBS (Leica Microsystems, Bensheim, Germany).

**Electron microscopy**

G12660 and control fibroblast cells were fixed, embedded in Epon and processed for electron microscopy according to standard procedures (80). Cells were incubated with the primary antibodies R27 and X223 (diluted 1:100 and 1:200 in PBS) for 1 h, the secondary antibody coupled to colloidal gold particles (6–12 nm; Dianova) was diluted 1:20 for ~6–12 h.

Photographs were taken with an electron microscope Zeiss EM 10 (Carl Zeiss).

**Construction of plasmids and mutagenesis**

To express an EGFP and hLMNA fusion protein within the point mutation hLMNA-p.S143F (C428T), the lamin A cDNA was amplified using a plasmid containing the hLMNA cDNA (GenBank accession no. NM-170707, gift from Albiena Todorova, University Hospital of Obstetrics and Gynecology, Sofia, Bulgaria). The sequence was subcloned into the vector pEGFP-C1 (Clontech, Heidelberg, Germany). Then the point mutation was introduced according to the protocol of the QuickChange® Multi Site-Directed Mutagenesis system (Stratagene, Amsterdam, Netherlands). The point mutants were obtained by using the following primers: S143F-forward: 5′-GGATGCCTCATGCTAAGGCGCTTTCCA-3′; S143R-reward: 5′-TGCTGCTGCTGCTGCTGGCTCGCTC-3′ (biomers.net, Ulm, Germany); which started directly in front and right behind of the relevant domain.

**Transfection**

The control skin fibroblasts were grown on Celllocate cover slips (Eppendorf, Hamburg, Germany) and transiently transfected with the EGFP-C1_S143F plasmid DNA or GFP-LamA plasmid DNA as control using Lipofectamine 2000 Transfection Reagent (Invitrogen) as specified by the manufacturer. Mouse wild-type dermal fibroblasts were transfected by electroporation (200 V, 950 μF; Gene Pulser Xcell, Bio-Rad). HaCaT cells were transfected by using the Amaxa nucleofector technology according to manufacturer’s instructions (Amaxa Biosystems). The transfected cells were grown for 12–20 h before further treatment.

**Morphometric analysis**

G12660 skin fibroblasts (passages 11 and 15) and control fibroblasts (passages 12 and 18) were subjected to indirect immunofluorescence using the nesprin-2 polyclonal pAb K1 and the monoclonal K20-478 antibodies. Nuclei were stained with DAPI. Nuclei with a smooth oval shape were assessed as normal, whereas nuclei harbouring blebs or gross irregularities in shape were assessed as abnormal. To analyse changes in nuclear shape, perimeter and area of nuclei of control skin fibroblasts and G12660 skin fibroblasts in different passages were measured. Measurements were performed in triplicate and 100 randomly chosen nuclei were measured to define the extent of nuclear deformation by calculating the nuclear contour ratio (4πarea/perimeter²) per experiment. The contour ratio for a circle is 1; when a nucleus shows increasing deformations, the contour ratio decreases.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG Online.

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Conflict of Interest statement
None declared.

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REFERENCES