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Research article

Expression and localization of nuclear proteins in autosomal-dominant Emery-Dreifuss muscular dystrophy with LMNA R377H mutation

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Abstract

Background: The autosomal dominant form of Emery-Dreifuss muscular dystrophy (AD-EDMD) is caused by mutations in the gene encoding for the lamins A and C (LMNA). Lamins are intermediate filament proteins which form the nuclear lamina underlying the inner nuclear membrane. We have studied the expression and the localization of nuclear envelope proteins in three different cell types and muscle tissue of an AD-EDMD patient carrying a point mutation R377H in the lamin A/C gene.

Results: Lymphoblastoid cells, skin fibroblasts, primary myoblasts and muscle thin sections were studied by immunocytochemistry and electron microscopy. Cellular levels of A-type lamins were reduced compared to control cells. In contrast, the amount of emerin and lamin B appeared unaltered. Cell synchronization experiments showed that the reduction of the cellular level of A-type lamin was due to instability of lamin A. By electron microscopy, we identified a proportion of nuclei with morphological alterations in lymphoblastoid cells, fibroblasts and mature muscle fibres. Immunofluorescence microscopy showed that a major population of the lamin B receptor (LBR), an inner nuclear membrane protein, was recovered in the cytoplasm in association with the ER. In addition, the intranuclear organization of the active form of RNA polymerase II was markedly different in cells of this AD-EDMD patient. This aberrant intranuclear distribution was specifically observed in muscle cells where the pathology of EDMD predominates.

Conclusions: From our results we conclude: Firstly, that structural alterations of the nuclei which are found only in a minor fraction of lymphoblastoid cells and mature muscle fibres are not sufficient to explain the clinical pathology of EDMD; Secondly, that wild type lamin A is required not only for the retention of LBR in the inner nuclear membrane but also for a correct localization of the transcriptionally active RNA pol II in muscle cells. We speculate that a rearrangement of the internal chromatin could lead to muscle-specific disease symptoms by interference with proper mRNA transcription.

Background

The lamins are a group of intermediate filament proteins which form major components of the nuclear lamina in most differentiated eukaryotic cells. The expression of lamins is developmentally regulated but most cell types in the adult body contain A- and B-type lamins (for review see [1]). In humans, three genes, LMNA, LMNB1 and LMNB2, encode distinct subtypes of proteins. The LMNA gene gives rise to lamins A and C by alternative splicing [2]. Lamins B1 and B2 are encoded by the two LMNB genes and are constitutively expressed independently of developmental stage. The expression of lamin B3, a splice variant of lamin B2, is limited to germ cells. In order to build up the nuclear lamina, lamins form homo- and heteropolymers which associate with other proteins into a network that underlies and supports the nuclear membrane (for review see [3]).

The lamins came into the focus of clinical interest when the LMNA gene was found to cause a rare heritable progressive myopathy, the autosomal-dominant form of Emery-Dreifuss muscular dystrophy (AD-EDMD, OMIM #181350; [4]). Another variant of this disease which is transmitted as an X-linked trait (X-EDMD, OMIM # 310300), had been earlier associated to mutations in emerin, a transmembrane protein of the inner nuclear membrane [5]. Thus, EDMD was the first disease found to be due to defects in proteins of the nuclear envelope. Clinically, the two variants are quite similar and are characterised by (1) a progressive muscular weakness with humeroperoneal distribution, (2) early contractures of the Achilles tendon, the elbows and the post-cervical muscles, and (3) atrial arrhythmias and/or a cardiomyopathy. A combination of these three cardinal symptoms is rarely seen in other myopathies and appears to be a discriminating feature of EDMD. Typically, symptoms develop in the second decade of life with the contractures often preceding clinically significant weakness. In young patients, the cardiac arrhythmia may go unnoticed and can lead to sudden death by cardiac arrest. Therefore, an early diagnosis is potentially life saving, since heart function can be stabilised by implantation of a cardiac pace maker [6].

In the past years, defects in the LMNA gene have been recognised to cause a pleiotropy of clinical phenotypes in 3 other autosomal dominant and 3 recessive disorders: (i) a form of limb-girdle muscular dystrophy with cardiac conduction defects (LGMD1B, OMIM #159001; [7]); (ii) a dilated cardiomyopathy with conduction defects (CMD1A; OMIM #115200; [8]), (iii) a familial partial lipodystrophy (FPLD; OMIM #151660; [9,10]); (iv) a recessive form of EDMD [11], (v) an autosomal recessive axonal neuropathy (Charcot-Marie-Tooth disease 2B1; CMT2B1; OMIM #605588; [12,13]) and (vi) mandibuloacral dysplasia (MAD; OMIM #248370, [14]). Recently,

dominant *de novo* mutations have been shown to cause the Hutchinson-Gilford progeria (HPGS; OMIM #176670, [15,16]). Furthermore, an association of a LMNA polymorphism to quantitative determinants of obesity was reported [17]. A review of the published mutations, mostly heterozygous amino acid replacements, suggested that interactions of specific domains of the lamin A/C protein with as yet unknown proteins may lead to the spectrum of tissue-specific mutations [11,18,19]. In the case of Emery-Dreifuss muscular dystrophy mutations are found mainly in the C-terminal domain of the protein leading to disturbance of dimerisation and fusion of the dimers to filaments [17,20]. A direct interaction between emerin and the lamins could be shown [21,22]. Moreover, mutations in either one of these proteins cause similar clinical symptoms indicating a strong interaction within a common function.

Despite numerous observations, up to now there is no conclusive explanation for the tissue-specific effect of the mutations in the emerin-lamin A/C complex. Although the proteins are expressed ubiquitously, mutations lead to primary cell damage and pathology in very specific cell types only whereas in most other tissues the mutations show no effect.

Progressive muscle wasting in EDMD is the result of a failure of adequate muscle regeneration. Muscle fibres destroyed by mechanical stress cannot be reconstituted by a sufficient amount of newly differentiated myotubes. This leads to a decrease of muscle mass and subsequent weakness [23]. According to one hypothesis, the function of the emerin-lamin A/C complex is the stabilisation of the nuclear envelope [24]. This may be of particular importance in cells which are subject to mechanical stress like muscle cells [25]. The disturbance of this stabilizing system and the resulting cell death in the affected muscle could, thus, explain the clinical symptoms of the disease.

The mutations in the lamin A-gene which have been analysed in detail [26] show different, partly position dependent effects like mislocalization of lamin A (R453W) or lamin C (E358K), and an apparent reduction of emerin expression in the nucleus (R527P; [26,27]). This could compromise the structural stability of the nucleus as a consequence of the altered lamina [28,29]. In other mutations like L530P, a wild-type distribution of lamin A, lamin C and emerin was observed [27]. These results could indicate that in this case another so far unknown function of lamin A and/or emerin is disturbed. Recently a role of lamin A and associated proteins in gene expression has been postulated [30]. Furthermore, cDNA microarray analysis has revealed changes in gene expression in X-EDMD fibroblasts [31].

We had the rare opportunity to study three different cell types from one AD-EDMD patient (99-3) carrying a point mutation in the rod domain of the LMNA gene which replaces arginine 377 by histidine (R377H). In the present study we used lymphoblasts, myoblasts, skin fibroblasts and muscle thin sections to examine the effects of this mutation on nuclear structure and proteins. Our results show that the mutation LMNA R377H causes defects in lamin A stability and assembly which can extend to an aberrant nuclear phenotype. We further demonstrate a mislocalization of LBR and RNA pol II in the patient's cells.

Results

The cellular level of the A-type lamins is reduced in cells of AD-EDMD patient 99-3

In the course of the diagnostic work-up, a Western blot of proteins from lymphoblastoid cells of the patient 99-3 showed a normal expression of emerin. A mutation in the emerin gene was excluded by subsequent sequencing (data not shown). Screening of the LMNA gene revealed a mutation in codon 377 (CGC > CAC) replacing Arg377 by His (LMNA R377H).

In order to study the expression level of nuclear lamin in this patient, we have separated nuclear proteins from different cell lines of controls and patient 99-3 by one-dimensional SDS-PAGE and transferred them to nitrocellulose (Fig. 1). In order to ensure that the lamin proteins have been well solubilized, the different samples were resuspended twice in the SDS-PAGE sample buffer. When nuclear proteins of the control cells were probed with antibodies against lamin A/C or B2 (lanes 1–3), the lamin antibodies reacted in all samples. In contrast, we found that in lymphoblastoid (lane 4) and fibroblast cells (lane 6) of the patient, the cellular level of lamin A/C was reduced (compare lanes 4, 6 with lanes 1, 3). Surprisingly, in myoblast cells (lane 5) and muscle tissue (lane 8) the level of lamin A/C appeared normal. The emerin antibody reacted in all nuclear extracts with a band of nearly uniform intensity indicating that equivalent amounts of protein had been loaded onto the different lanes (lanes 1–8). The same blot was re-probed with anti-fibrillarin antibodies to ensure equal protein loading (lanes 1–6 insert). We further studied the three cell types by immunofluorescence microscopy. When lymphoblastoid cells with the mutation LMNA R377H were labelled with the antibodies against emerin (Fig. 2a) or lamin B2 (Fig. 2c), all nuclei revealed the typical prominent staining of the nuclear periphery. However, after incubation with the anti-lamin A/C antibody, we found a deficiency of the immunostaining in 90% of nuclei (5121 out of 5690, Fig. 2b), while control lymphoblastoid cells showed clearly positive immunostaining of the nuclear membrane with all antibodies used (Fig. 2d,2e,2f). This result is in agreement

with the immunoblot data, where lymphoblastoid cells showed a clear reduction of the lamin A/C signal.

In order to address potential tissue-specific effects of the LMNA R377H mutation we studied lamin A/C levels in other cell types from the same patient. Immunofluorescence studies of emerin, lamin A/C and lamin B on fibroblast cells revealed a pattern of intense staining at the periphery of the cell nucleus after incubation with emerin or lamin B antibodies (Fig. 3a,3c) as previously described for the lymphoblastoid cells (Fig. 2a,2c). However, we observed a difference in the staining of lamin A/C. Whereas nearly all lymphoblastoid nuclei showed a reduced or absent staining (Fig. 2b) only approximately 20% of the fibroblast nuclei (614 out of 3080) were not labelled by the lamin A/C antibody (Fig. 3b). As expected, the fluorescence of control fibroblasts showed an intense staining of the nuclear envelope with all antibodies used (Fig. 3d,3e,3f).

In the immunoblot experiments we had detected a reduced amount of lamin A/C in lymphoblastoid and fibroblast cells but surprisingly not in myoblasts and mature muscle (see Fig. 1). However, by immunofluorescence microscopy of myoblasts (Fig. 4) or frozen sections of a muscle biopsy of patient 99-3 (Fig. 5), about 5% (169 out of 3290) of myoblast nuclei (Fig. 4b) and 5% (79 out of 1580) of mature muscle nuclei showed only a faint staining with lamin A/C antibodies (Fig. 5b, arrow). In contrast, we never saw a reduction of lamin A/C staining in control myoblasts (3430 nuclei) or mature muscle (1650 nuclei). Nuclear periphery staining with emerin or lamin B2 antibodies was normal (Figs. 4, 5a,5c) as was the staining of control myoblasts and muscle sections by all antibodies (Figs. 4, 5d,5e,5f). It is interesting to note that the reduced amount of lamin A/C has been observed not only in cultured cells (Figs. 2, 3, 4), but also on frozen muscle sections (Fig. 5). This indicates that the observed reduction of lamin A/C staining is not caused by our cell culture conditions. Given that only 5% of nuclei in myoblasts and mature muscle fibres showed a reduction of lamin A/C staining it is not surprising that immunoblotting failed to demonstrate a reduction of the level of lamin A/C by (Fig. 1, lane 5).

These results support the interpretation that the cellular level of lamin A/C is reduced in cells carrying a R377H mutation in the LMNA gene. For the first time, the reduction of lamin A/C could be demonstrated by two independent methods, i.e. Western blotting and immunofluorescence microscopy. Nuclear alterations in muscle cells of an AD-EDMD have been described before [32], however, the immunohistochemical reactions with anti-lamin A/C antibodies were normal in this study.
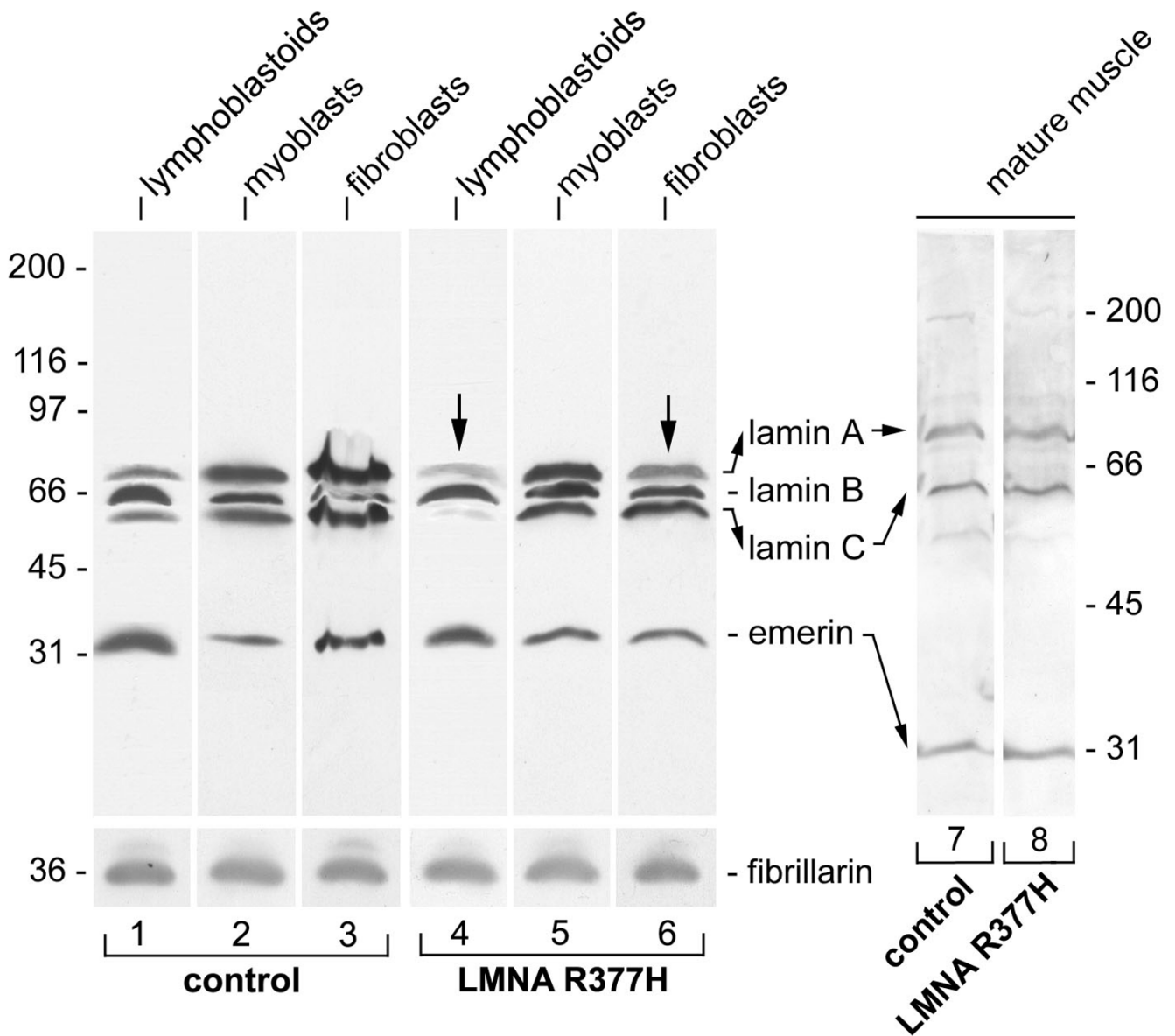
Figure 1
A-type lamins are reduced in cells with the mutation LMNA R377H. To determine the level of lamin A/C, lamin B and emerin in AD-EDMD patient 99-3, whole proteins of cell extracts from patient lymphoblastoid (lane 4), myoblast (lane 5), fibroblast (lane 6) cultures and mature muscle (lane 8) or control lymphoblastoid (lane1), myoblast (lane 2) fibroblast (lane 3) cultures and mature muscle (lane 7) were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with mab R27 to detect lamin A/C, mab X223 to detect lamin B2, mab NCL-emerin to detect emerin and PEG3 to detect fibrillarin. The same nitrocellulose was used for all antibodies. The antibodies were stripped before the incubation with the next one. The level of lamin A is reduced in lymphoblastoids and fibroblasts cells (see arrows in lanes 4 and 6). Molecular mass standards are indicated in kDa.
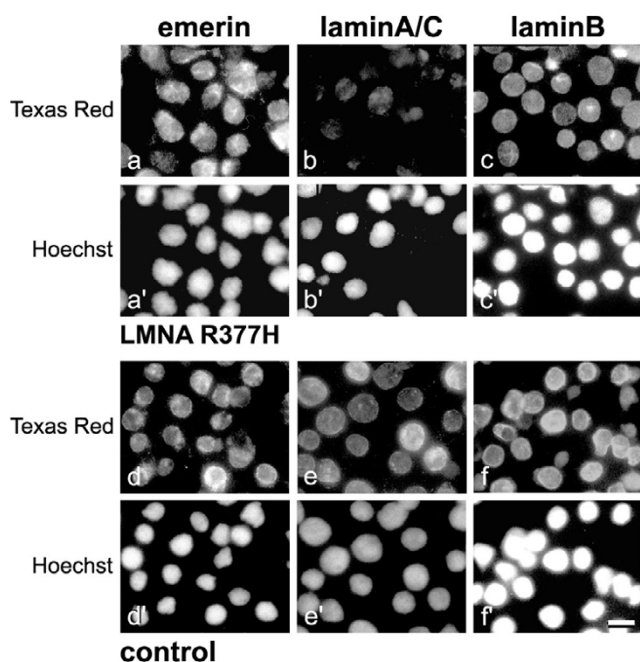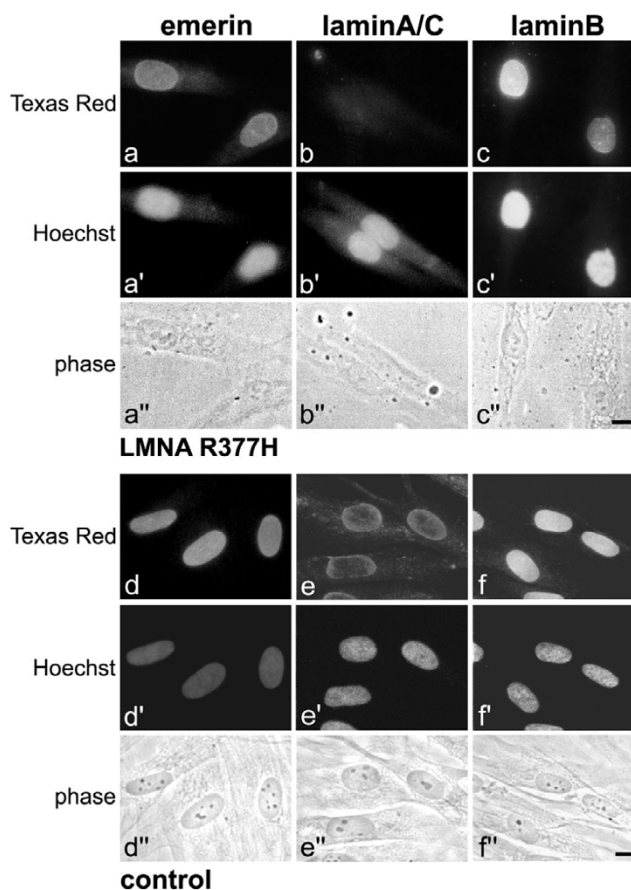
Figure 2
Immunostaining for lamin A/C is deficient in lymphoblastoid cells with mutation LMNA R377H. Indirect immunofluorescence microscopy of lymphoblastoid cells from patient cells (a-c) and control cells (d-f) after staining with antibodies against emerin (a,d), lamin A/C (b,e) and lamin B (c,f). Lamin A/C staining is less intense in patient cells (b) compared with the control (e). The corresponding Hoechst fluorescence in shown (a'-c' and d'-f'). Bar: 10 μm.

In total, we have screened lymphoblastoid cells from another 14 patients clinically diagnosed with AD-EDMD, but we detected reduced levels of lamin A/C only in patient 99-3 and one other patient with an as yet uncharacterized genetic defect (data not shown).

A-type lamin is unstable in cells with the mutation LMNA R377H

In order to study the stability of the mutated lamin A/C, lymphoblastoid cells of patient 99-3 were blocked in the G1 phase by incubation in medium with 10 μM lovastatin [33]. The arrest in the G1 phase was checked by FACS analysis (data not shown). After G1 phase arrest it is possible to analyze the stability of proteins during a defined time period. The level of lamin A/C expression in G1-arrested patient cells was assayed on immunoblots and compared to G1 arrested control cells. In control cells, the level of lamin A/C was stable in the presence of lovastatin over an incubation period of up to 48 h. (Fig. 6A, lanes 1–3). In patient cells, the amount of lamin A was considerably reduced by incubation with lovastatin (Fig. 6A, lanes

Figure 3
Some fibroblast cell nuclei from the AD-EDMD patient 99-3 are negative for antibodies to lamin A/C. Immunolabelling of fibroblast cells with mutation LMNA R377H (a-c) and control cells (d-f) was done by using antibodies against emerin (a,d), lamin A/C (b,e) and lamin B (c,f). Not all nuclei stain for lamin A/C in patient cells (compare b with e). The corresponding phase-contrast images (a"-c"; d"-f") and Hoechst fluorescence (a'-c'; d'-f') are shown. Bars (c",f") 10 μm.

4–6) while the amounts of lamin C and emerin appeared only slightly reduced. This reduction is not due to an inhibition of processing [34]. If so, our antibody would also recognize the pre-lamin A. Similar results were obtained after incubation with the protein synthesis inhibitor cycloheximide (50 μg/ml). The amount of lamin A and C was clearly reduced in the patient sample (compare Fig. 6B lanes 3 and 4). These data, in combination with the observation of the reduced levels of lamin A by immunoblotting and immunofluorescence microscopy support the hypothesis that the mutation R377H leads to a shorter half life of lamin A.
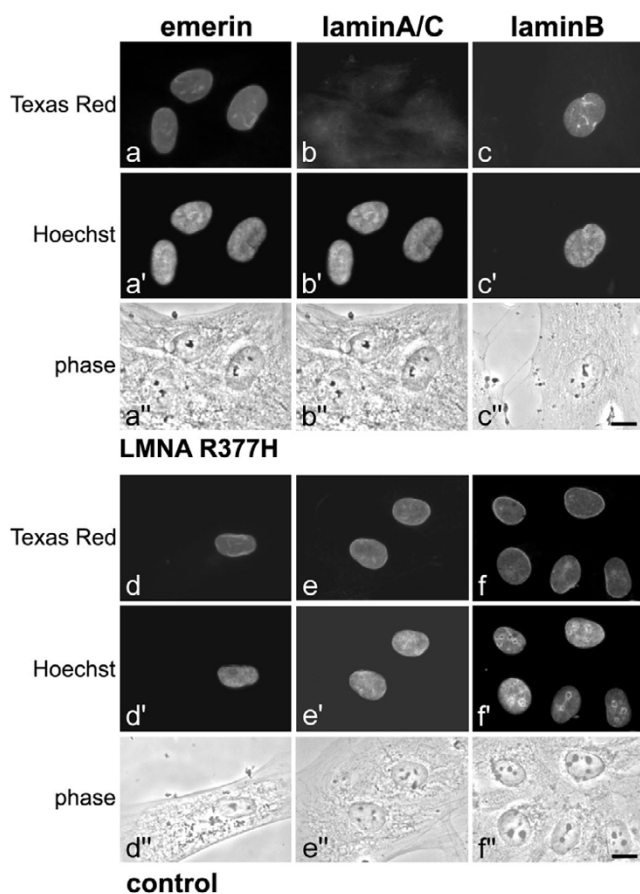
Figure 4
Some myoblasts cell nuclei from the AD-EDMD patient 99-3 are negative for antibodies to lamin A/C. Immunolabelling of myoblasts cells with mutation LMNA R377H (a-c) and control cells (d-f) was done by using antibodies against emerin (a,d), lamin A/C (b,e) and lamin B (c,f). Not all nuclei stain for lamin A/C in patient cells (compare b with e). The corresponding phase-contrast images (a"-c"; d"-f") and Hoechst fluorescences (a'-c'; d'-f') are shown. Bars (c",f") 10 μm.

Figure 5
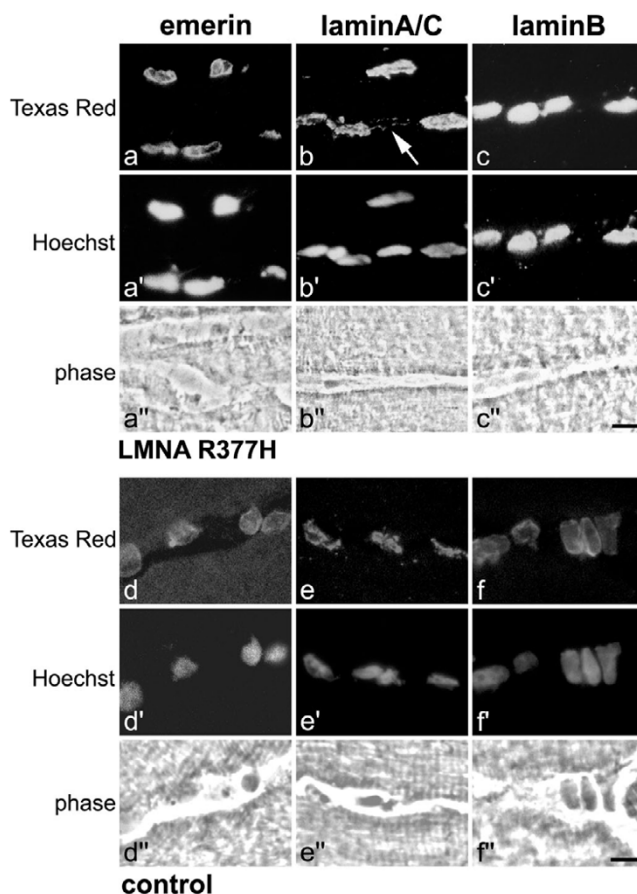**Immunostaining for lamin A/C is deficient in frozen muscle sections of the AD-EDMD patient 99-3**. Immunolabelling of frozen muscle sections from patient 99-3 (a-c) and control (d-f) was performed for emerin (a,d), lamin A/C (b,e) and lamin B (c,f). Lamin A/C staining is less intense in patient (arrow in b) compared with the control (e). The corresponding phase-contrast images (a"-c"; d"-f") and Hoechst fluorescences (a'-c'; d'-f') are shown. Bars (c",f") 10 μm.

*Nuclear alterations in cells of the AD-EDMD patient 99-3*
It has been previously described that about 10% of muscle cell nuclei from AD-EDMD patients show an alteration of nuclear morphology [32,35]. To examine their ultrastructure more closely, nuclei of lymphoblastoid cells (875), fibroblasts (520), myoblasts (490), and mature muscle fibres (375) from patient 99-3 and controls were analysed by electron microscopy. In lymphoblastoid cells with the mutation LMNA R377H, a striking alteration of nuclear morphology was observed. While the nuclei of control lymphoblastoid cells display a normal morphology (Fig. 7a,7a'), the nuclei of the patient cells were surrounded by a continuous double membrane with numerous blebs and the peripheral heterochromatin was no longer associated with the inner nuclear membrane (Fig. 7b, arrows). Due to the chromatin condensation, the inner pore fibrils became visible (Fig 7b', arrows). Such nuclear alterations were found in about 40% of the patient's lymphoblastoid cells. However, the cells proliferated normally indicating that the observed morphological changes are not indicative of apoptosis.

In fibroblasts (Fig. 7d,7d'), myoblasts (data not shown) and muscle sections (Fig. 7f,7f') some nuclei showed nuclear membrane invaginations (Fig. 7d',7f'), while the
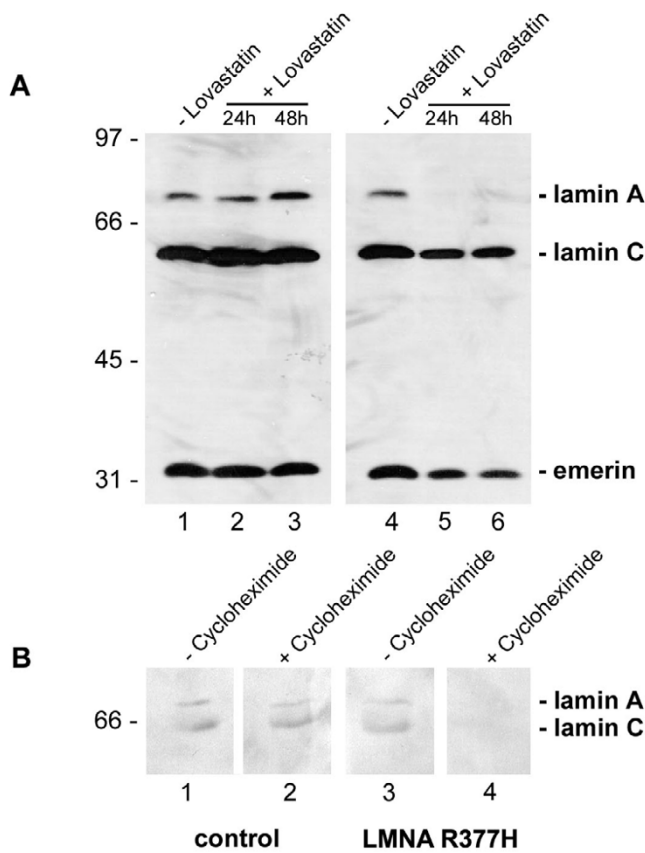
Figure 6
Stability profiles of lamin A/C and emerin in lymphoblastoid cells culture after treatment with lovastatin or cycloheximide. To determine the stability profiles of lamin A/C and emerin, cells were grown in the presence or absence of lovastatin (panel A) or cycloheximide (panel B). In A, lymphoblastoid cells with the mutation LMNA R377H (lanes 4–6) and control (lanes 1–3) were grown in the presence of 10 µM lovastatin for 24 h (lanes 2, 5), for 48 h (lanes 3, 6) or in the absence of lovastatin (lanes 1, 4). In B, lymphoblastoid cells with the mutation LMNA R377H (lane 4) and control (lane 2) were grown in the absence (lanes 1, 3) or presence of 50 µg/ml of cycloheximide (lanes 2, 4). Whole cell extracts were resolved on SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with the mab R27 to detect lamins A/C and with the mAb NCL-emerin to detect emerin on the same stripped nitrocellulose. Significantly, lamin A from AD-EDMD patient is instable (compare panel A lanes 5, 6 with lanes 2, 3 and also panel B lane 4 with lane 2). Molecular mass standards are indicated in kDa.
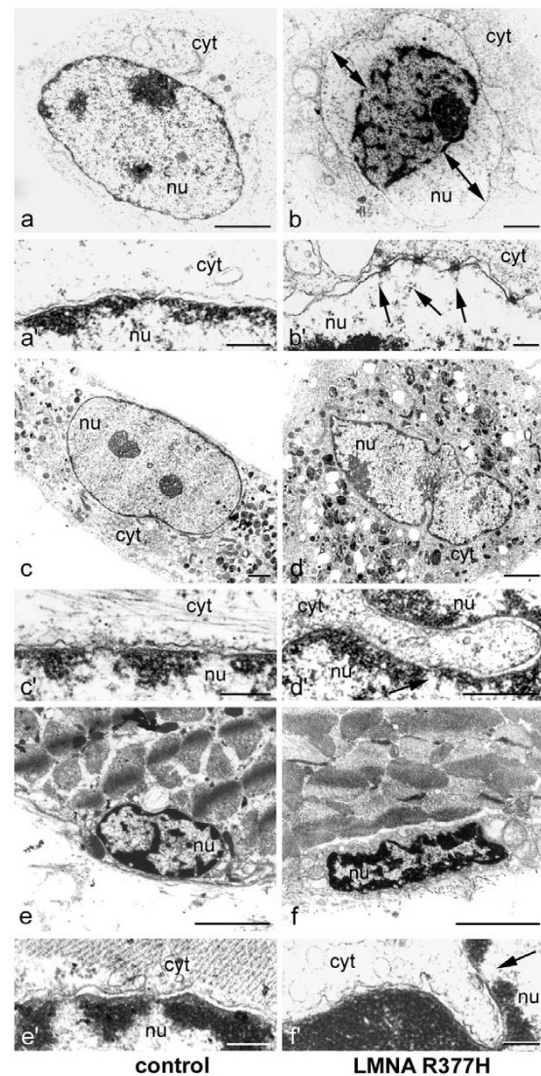
Figure 7
Electron microscopic examination of the nuclear morphology in AD-EDMD patient cells. Electron microscopic analysis of lymphoblastoids (a-b'), fibroblasts (c-d') and frozen muscle sections (e-f') from AD-EDMD patient 99-3 (b,b',d,d',f,f') and from control (a,a',c,c',e,e'). In the control a largely continuous layer of heterochromatin is in contact with the inner face of the nuclear envelope [visible at low (a,c,e) and higher magnification (a',c',e')]. In the lymphoblastoid cells with mutation LMNA R377H the condensed peripheral heterochromatin appears clearly detached from the nuclear envelope (arrows in b), the double membrane surrounding the chromatin contains numerous blebs and the inner pore fibrils become visible as shown at higher magnification (b', arrows). In patient fibroblast cells and muscle sections, nuclei showed nuclear membrane invaginations while the morphology of the membrane and nuclear pores was not disturbed as shown at higher magnification (d',f', arrows). In patient mature muscle cells, condensation of the peripheral chromatin was observed (f) compared to control (e). Bars (a-f): 2 µm; (a'-f') 0.2 µm.

morphology of the membrane and the nuclear pores was not disturbed (Fig. 7d',7f' arrows). Such invaginations were found in 10% of nuclei studied, but never observed in control cells (Fig. 7c',7e'). These membrane invaginations seem to be different from both the large intranuclear channel system previously reported for muscle nuclei of X-EDMD patients and the deep invaginations of the nuclear membrane observed in an AD-EDMD patient [35]. In muscle sections, the peripheral chromatin appeared more condensed compared to controls (compare Fig. 7e and 7f). This chromatin condensation was found in 20% of the nuclei studied.

Distribution of LBR in different cell types from patient 99-3

Since a selective retention is required for the localization of integral proteins in the inner nuclear membrane [36-38], it is possible that A-type lamins are involved in this process. Therefore, we have studied the distribution of several inner nuclear membrane proteins in different cell types from patient 99-3 and in control cells by immunocytochemistry. Emerin (Figs. 2a, 3a, 4a, 5a and 2d, 3d, 4d, 5d), LAP2β (data not shown; [39,40]), and MAN1 (data not shown; [41]) showed a normal localization at the nuclear rim in all cell types. The presence of emerin in the nuclear envelope indicates that the interaction of emerin with the lamina is not directly affected by the mutation LMNA R377H, localized in the rod domain, as has been previously shown [26]. Surprisingly, immunofluorescence microscopy revealed differences in the subcellular localization of the lamin B receptor. LBR possesses 8 membrane spanning domains and is a potential binding partner for nuclear ligands like lamin B, human chromodomain protein HP1, and DNA (see review [42]). In control fibroblasts and myoblasts, LBR is highly enriched at the nuclear envelope (Fig. 8a',8c'). The same holds true for lymphoblastoid cells (data not shown). In about 95% of patient cells (2560 fibroblasts, 2840 myoblasts and 4326 lymphoblastoids), a large fraction of LBR immunostaining is found in the cytoplasm with a distribution similar to ER proteins (Fig. 8b',8d'). This was verified by double immunofluorescence using ribophorin II antibodies (data not shown). After double labelling with anti lamin A/C and anti LBR in patient fibroblasts and myoblasts (Fig. 8e",8f") we could show that the absence of lamin A/C correlates with the location of LBR to the ER (Fig. 8e,8e"). However, due to the fact that in 95% of patient cells (see above) a large fraction of LBR was found in the cytoplasm but that a lamin A/C deficiency was observed only in 5 to 20% of nuclei (myoblasts and fibroblasts, respectively), our data suggest that not the absence but the structural changes in the mutated lamin are inducing the anomalous location of LBR. While the overall levels of LBR in patient and control cells were identical when compared by immunoblotting with anti LBR antibodies (data not shown), a considerable amount of LBR is no longer retained within the inner nuclear membrane, but probably diffuses to the ER via the membrane continuities at the periphery of the nuclear pore complexes [43]. However, lamin B2 still concentrates within the nuclear envelope (Fig. 8 compare a,c with b,d). These results indicate that the mislocalization of LBR correlates with the expression of the mutated lamin A.

Differences in RNA pol II localization in myoblast cells with the mutation LMNA R377H

It has been reported that lamins and lamin-associated proteins bind to chromatin and that the disruption of nuclear lamin polymers inhibits RNA pol II-dependent transcription [30]. Since our data indicated that the LMNA R377H mutation impairs lamina stability we examined the distribution of phosphorylated RNA pol II in our patients and control cells. Immunofluorescence with RNA pol II antibodies revealed a normal, characteristic pattern of nuclear speckles not only in control fibroblasts (Fig. 9a") and myoblasts (Fig. 10a") (2970 and 2765 cells observed respectively) but also in fibroblast cells of patient 99-3 (Fig. 9b") (3240 cells observed). However, the distribution of phosphorylated RNA pol II was altered in 75% (1981 of 2643) of myoblasts with the mutation LMNA R377H and appeared to concentrate at the poles of the nuclei (Fig. 10b",10c", arrows). After double labelling of patient myoblasts with anti lamin A/C and anti RNA pol II (V/22) we could show that the absence or reduction of lamin A/C correlates with the altered distribution of phosphorylated RNA pol II (Fig. 10e,10e"). Similar to the results with LBR (see above), the dramatic alteration of RNA pol II distribution observed in 75% of the myoblasts compared to the reduced level of lamin A/C observed in only 5% of myoblasts suggests that the relocalization of RNA pol II within nuclei of muscle cells is a direct or indirect consequence of not only the absence but also of structural changes in lamin A. Furthermore, the lamin A mutant R377H seems to influence exclusively the localization of the phosphorylated form of RNA pol II, since the distribution of the unphosphorylated, inactive form of RNA pol II was identical to that in controls (Fig. 10d", compare to a").

Discussion

LMNA R377H induces abnormalities in the nuclear envelope

In this study, we describe the effects of a defective nuclear lamin A in cells derived from AD-EDMD patient 99-3. Genomic sequencing of all the LMNA gene's exons identified a mutation in exon 6, replacing arginine 377 by histidine. The R377H mutation has been reported before in a family with limb girdle muscular dystrophy 1B [7]. Like a number of other mutations it is localized in the helical rod region. By Western blotting and immunofluorescence
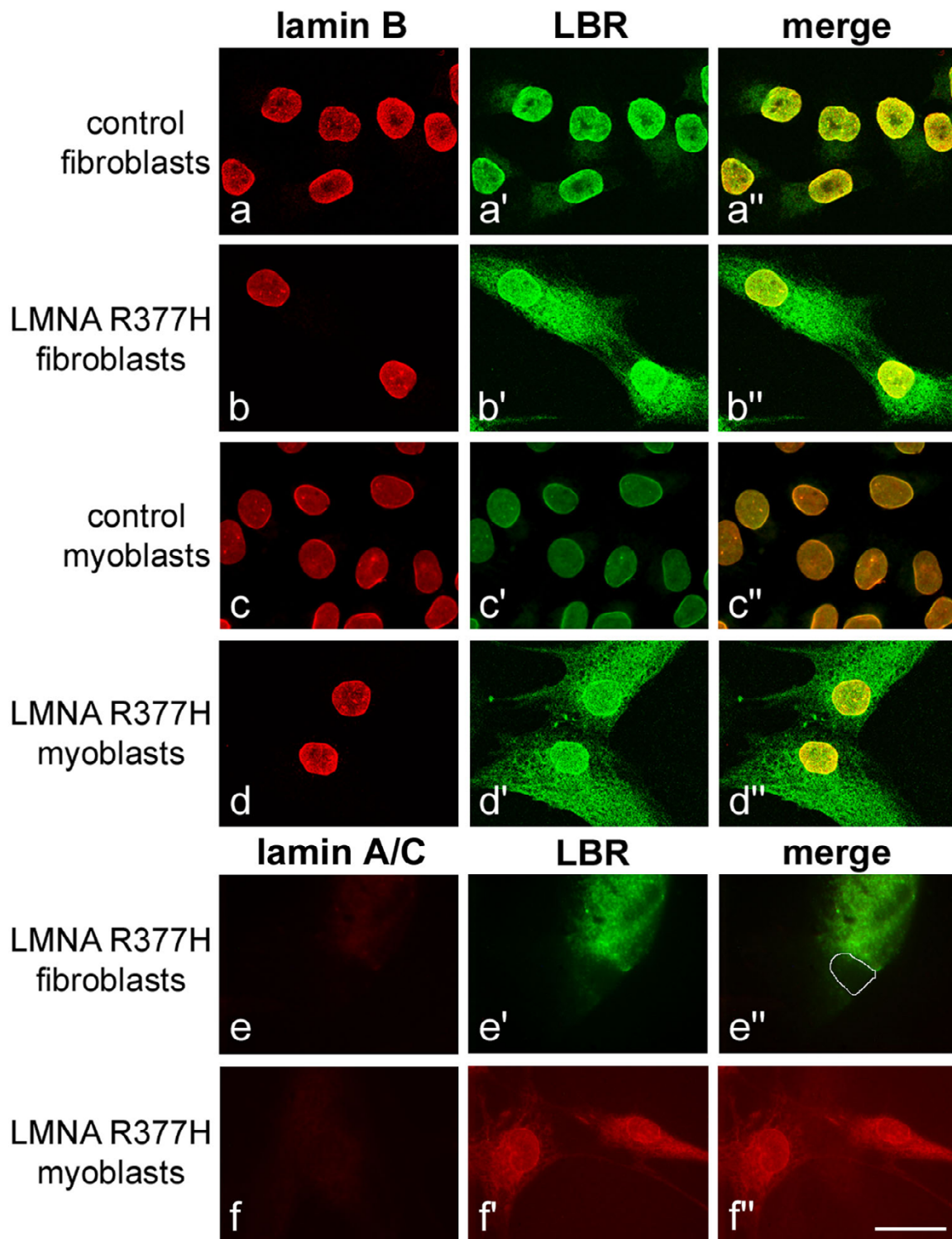
Figure 8
Mislocalization of LBR in cells with mutation LMNA R377H. Fibroblasts (a,b,e) and myoblasts (c,d,f) from control (a,c) or patient (b,d,e,f) were double labelled with a mAb directed against lamin B2 (a-d) or lamin A/C (e,f) and with guinea pig antibodies recognizing LBR (a'-f') and then analyzed by confocal microscopy. Note the colocalization of lamin B and LBR at the nuclear envelope in control cells (a'',c''). In contrast, in patient cells LBR shows also a cytoplasmic localization (b'',d'',e'',f''). Merged pictures are shown in a''-f''. The contour of the nucleus is marked by a white line (e''). Bar: 15 μm.
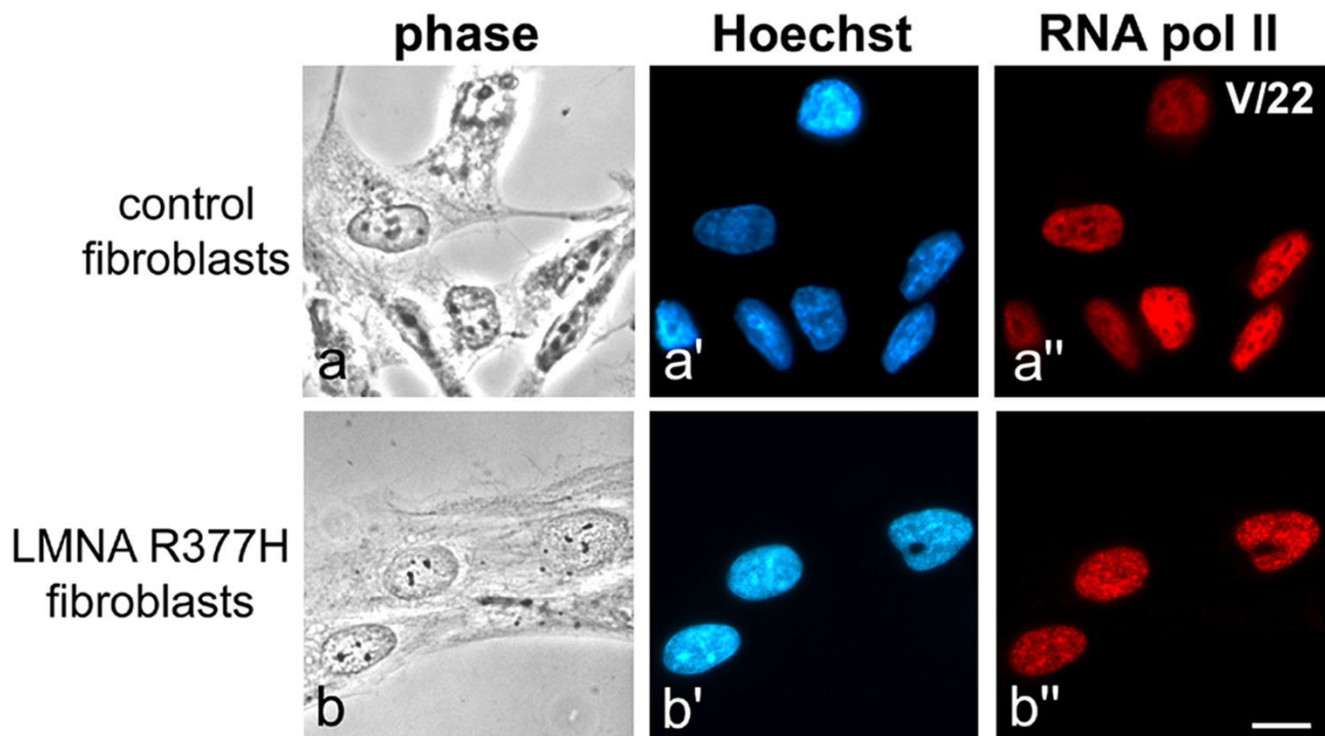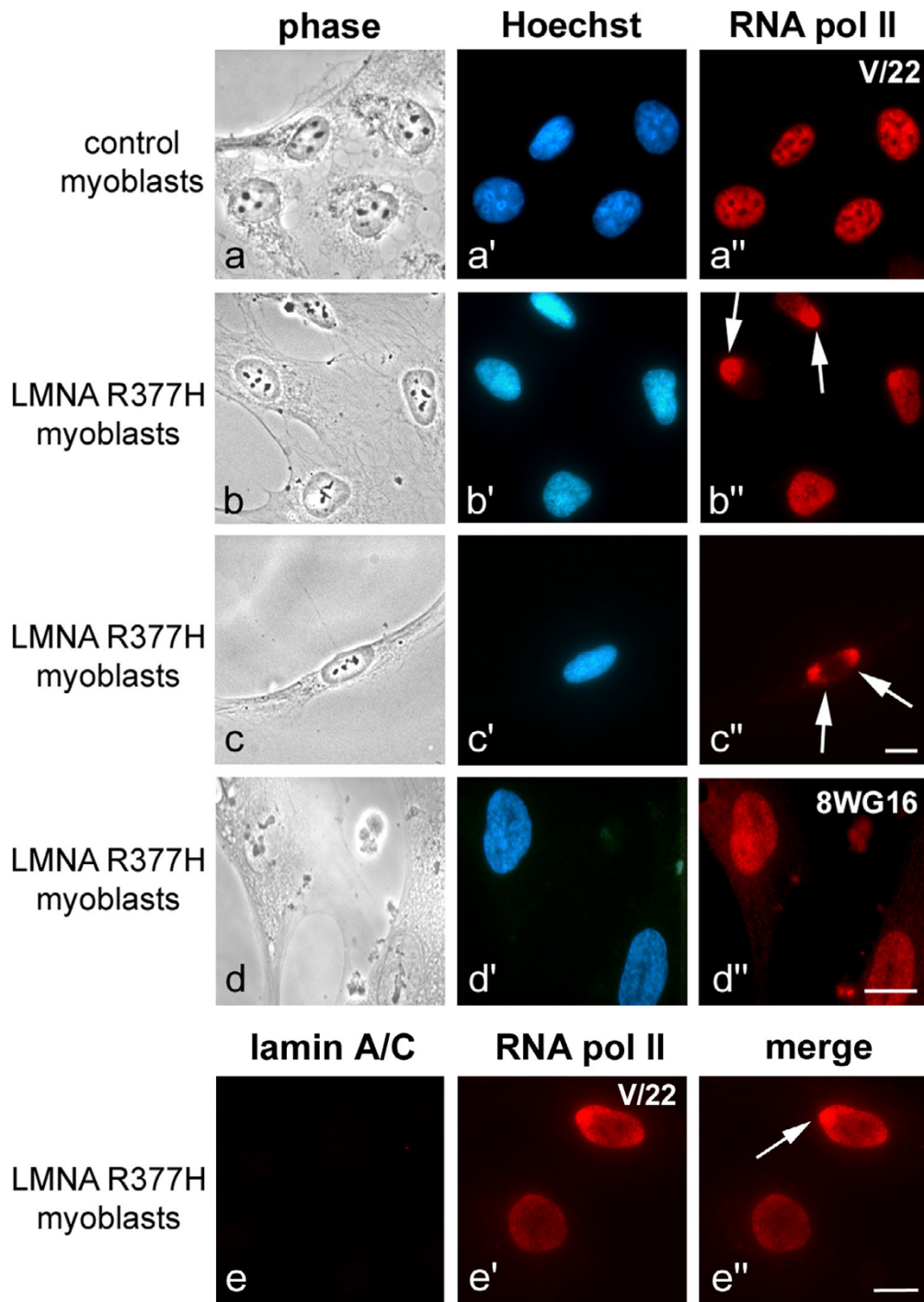
Figure 9
Normal distribution of the phosphorylated RNA pol II in fibroblast nuclei of AD-EDMD patient 99-3. Fibroblast culture cells from control (a) or patient (b) were immunolabelled with mab V/22 recognizing RNA pol II (a",b"). Note the normal distribution of RNA pol II all over the nucleus in control as well as in patient fibroblasts (compare a" and b"). The corresponding phase-contrast images (a,b) and Hoechst fluorescences (a',b') are shown. Bar: 10 μm.

microscopy, it was possible to demonstrate for the first time that this mutation leads to a reduction of the A-type lamin level. A less intensive staining for lamin A/C had already been reported after immunolabelling of frozen muscle and heart sections of AD-EDMD patients [24,44] as well as in cultured skin cells of an X-EDMD patient [45]. But until now no reduction of A-type lamin was observed by protein analysis using Western blotting. The reduction of the total lamin A level is indicative of this mutation rendering lamin A less stable. In a dominant disease, 50% of normal lamin A/C may be expected from the wildtype allele while the affected allele may produce another 50% of an altered protein. Our results suggest that mutated lamin A/C is unstable and rapidly degraded. During G1 phase arrest induced by the drug lovastatin or after protein synthesis inhibition by cycloheximide, the amount of A-type lamin in cells with the mutation R377H was decreased compared to control cells. Previous studies have reported an increased solubility of lamins in skin fibroblasts from an X-EDMD patient [46]. In a recent report Vigouroux and co-workers [47] showed that a LMNA R482Q/W mutation in fibroblasts from lipodys-

trophic patients does not affect the nuclear content in lamins, but increases their extractability. On the other hand Östlund et al. [48] showed, after pulse-chase analysis, that several other lamin A mutants were as stable as wild-type lamin A. The difference between our findings and those reported by Markiewicz et al. [46], Vigouroux et al. [47] and Östlund et al [48] could rather reflect the effect of different lamin mutations than differences between AD-EDMD and X-EDMD or lipodystrophy. All lamins, i.e. B1, B2, A and C form dimers that assemble into multimeric filaments to form the nuclear lamina (see review [3]). For this process a functional rod domain is necessary [49]. The point mutation R377H is localized in the rod domain and could specifically change the 3D structure of the lamin A, disrupt intermolecular interactions involved in the normal dimerisation process, and thus affect the stability of the lamin network.

Previous histological and histochemical studies described dystrophic features in muscles from an AD-EDMD patient [44,35]. In this study, we found that the LMNA R377H mutation has an impact not only on lamin A stability but

Figure 10
Uneven distribution of the phosphorylated RNA pol II in myoblast nuclei from AD-EDMD patient 99-3. Myoblast culture cells from control (a)or patient (b-e") were immunolabelled with mab V/22 recognizing the phosphorylated RNA pol II (a"-c") or with 8WG16 recognizing the unphosphorylated RNA pol II (d"). The patients myoblasts were double labelled with a polyclonal anti-lamin A/C antibody (e) and V/22 (e') and then analyzed by confocal microscopy. The merged picture is shown in (e"). Note that the dramatic concentration of phosphorylated RNA pol II is observed at the poles of the nuclei in patient cells (b",c",e" arrows). Note the normal localization of the phosphorylated RNA pol II in control cells (compare a" with b",c" and e") and of the unphosphorylated RNA pol II (d"). The corresponding phase-contrast images (a-d) and Hoechst fluorescences (a'-d') are shown. Bars: 10 μm.

also on nuclear architecture. In patient lymphoblastoid cells, the nuclei showed a condensation of peripheral heterochromatin. In addition, we observed a damaged nuclear membrane with numerous blebs. Abnormal nuclear morphology of muscle and skin cells were previously observed not only in X-EDMD patients [45,50] but also in an AD-EDMD patient [32]. It is interesting to note that damage of nuclear membranes with numerous blebs were only observed in cells of X-EDMD patients [45,50]. However, similar ultrastructural abnormalities have been described in fibroblasts and hepatocytes from mice lacking A-type lamins [51]. Structural abnormalities were also seen in about 10 to 20% of our patient's muscle and fibroblast nuclei. However, the nuclear membrane inclusions as well as the peripheral chromatin condensation observed in our AD-EDMD patient were different from the deep invaginations of the nuclear envelope producing pseudoinclusions reported by the group of Fidzianska who also observed strong alterations in chromatin organization in an AD-EDMD patient with a LMNA R453W mutation [35]. Taken together, both studies suggest that different mutations in LMNA may cause different morphological abnormalities in nuclei. It is tempting to correlate this to the wide spectrum of clinical phenotypes associated with LMNA gene mutations [52].

The abnormality of the nuclear envelope in mutated myoblasts was also reflected by the different permeabilization conditions required for the immunolabelling of control and patient myoblasts. A staining of the nuclei and nuclear envelope from patient myoblasts was obtained when cells were permeabilized for only 2 min in -20°C methanol:aceton (1:1). In contrast, control myoblasts required at least 20 min of permeabilization. This observation indicates that the nuclear envelope of the patient's cells is more permissive for antibodies probably due to reduced stability of the lamina. Indeed, it has been previously described that an intact lamina is essential for the correct and stable reassembly of membrane vesicles in forming a nuclear envelope [28,29].

From published reports [32,35,45] and our own observations it appears that the observed nuclear alterations affect a minority of nuclei and are not tissue specific. It is, therefore, hard to conceive that the structural changes alone can cause the pathology of LMNA defects. In the case of our patient this nuclear alteration is probably due to a reduced stability of A-type lamin. In our previous work, we were able to demonstrate that an intact lamina is essential for the correct and stable reassembly of an intact nuclear double membrane around the daughter nuclei after mitosis [28,29].

Other authors have demonstrated that emerin is strongly associated with the nuclear lamina [53,54] and the data of

Vaughan et al. [27], Harborth et al. [55], Östlund et al. [48] and Raharjo et al. [56] suggest that lamin A is essential for anchorage of emerin to the inner nuclear membrane. A direct interaction between recombinant emerin and lamin A has been shown *in vitro* [21]. However, it was also reported that an altered lamin distribution can occur without relocalization of emerin in FPLD fibroblasts carrying R482Q, R482W or R482L mutations [47,57]. In accordance with these data, emerin appeared correctly localized in the nuclear membrane in cells with the LMNA R377H mutation as shown by our immunolabelling experiments. In none of our experiments did the LMNA R377H mutation have an apparent influence on emerin.

In summary, the data suggest that different amino acid substitutions elicit different effects on the intermolecular interactions of A-type lamins and that their cellular level does not play a central role in retention of emerin in the nuclear membrane. Probably only a small amount of lamin A is required to immobilize emerin into the nuclear envelope. Indeed, Harborth et al. [55] were able to demonstrate that after lamin A/C silencing, emerin re-distributed to the cytoplasm.

Evidence for influences of LMNA R377H on chromatin organization

A set of integral nuclear proteins is known to interact with the lamina. Thus, the retention of these proteins at the inner nuclear membrane could be mediated by their binding to lamins (for review see [58]). For example, the lamin B receptor (LBR) and the lamina associated protein 2β (LAP2β) interact more specifically with B-type lamins [40,59], whereas emerin preferentially binds to A-type lamins [21,22,51]. In this study, we present evidence for a marked change of LBR localization in cells with the LMNA R377H mutation. In control cells, LBR is concentrated within the nuclear envelope, whereas in LMNA R377H cells there is a partial loss of nuclear envelope associated LBR, with a more general cytoplasmic distribution identical to that observed for ER proteins. Presumably LBR is no longer retained in the inner nuclear membrane and diffuses into the interconnected ER membrane system [37]. These results indicate that, at least in the cells with a LMNA R377H mutation, correct LBR localization is contingent upon the level of wild-type lamin A expression. However, it has been shown that treatment of HL-60 cells with retinoic acid decreased lamin A/C to negligible amounts but LBR was still localized in the inner nuclear membrane [60]. In *Xenopus* oocytes, peripheral chromatin but no lamin is required for the retention of LBR in the inner nuclear membrane [61]. Indeed, the nucleoplasmic domain of LBR binds not only to B-type lamins [62,63] but also to DNA [62] and chromosome/chromatin domains [64,65] besides interacting with human chromodomain proteins [66,67]. Our data support the hypothe-

sis that probably an as yet unknown chromatin protein associated to A-type lamin is involved in the retention of LBR in the nuclear envelope, and that in cells containing the probably dominant LMNA R377H mutation, this peripheral chromatin protein is no longer able to bind to the mutated A-type lamin and thus fails to establish the interaction with LBR.

Several roles have been proposed for the lamins, e.g. in nuclear envelope assembly, nuclear architecture, maintenance of chromatin organisation and DNA replication [28,29,68-70]. The implication of lamins being involved in the transcription process arose from the observation that in cells with a disrupted lamin organization, RNA polymerase II dependent transcription is dramatically impaired [30,57]. It has also been suggested that lamin A might affect gene expression by influencing the spatial organization of chromatin [71,72]. Our results support such data by demonstrating an abnormal accumulation of the phosphorylated form of RNA polymerase II which concentrates at the poles of the nuclei in myoblasts containing the LMNA R377H mutation, correlating with the failure of lamin A/C to assemble correctly.

An abnormal cytoplasmic accumulation of the RNA pol II largest subunit has been previously described in sporadic inclusion-body myositis, a progressive degenerative muscle disease of older age [73]. In our study, we present the first evidence for a marked difference in intranuclear organization of the phosphorylated RNA polymerase II in cells from an AD-EDMD patient. The disorganization was specifically observed in muscle cells where the pathology of EDMD predominates and where we also found nuclear membrane inclusions. This observed nuclear change is in accord with the nuclear chromatin reorganization described in AD-EDMD by Fidzianska et al. [35].

Conclusions
We speculate that the rearrangement of internal chromatin, provoked by mutant lamins, could lead to muscle-specific disease symptoms by interference with proper mRNA transcription. Indeed, transcription requires the hyper-phosphorylated form of RNA pol II [74]. In this context, an important observation was made by Tsuhakara et al. [31] who reported an altered gene expression profile after cDNA microarray analysis of X-EDMD fibroblast cells lacking emerin. Thus, evidence is accumulating that the peripheral lamina is not only important for maintaining nuclear integrity but may also play a role in the arrangement of chromatin for proper gene expression. Perhaps this function of the lamina may hold the key to understanding the tissue-specific effects of lamin A/C mutations.

Methods
Patient and cells
The diagnosis of the AD-EDMD affected patient 99-3 was based on clinical findings and DNA analysis. A specific mutation in the lamin A gene was identified by direct sequencing (LMNA c.1130G>A, R377H). For a histological diagnosis, a muscle biopsy was taken from the *vastus lateralis* [75]. In accordance with the ethical regulations of the hospital, the patient gave informed consent for further studies on his tissues. Lymphoblastoid and skin fibroblast cells (SV80 fibroblasts for control) were cultured in DMEM (Dulbecco's Modified Minimum Essential Medium) with 10% FCS and 1% L-glutamine. Myoblasts were cultured in DMEM high glucose with 20% FCS and 1% L-glutamine. Cells were grown at 37°C in a 5% CO_2 incubator. Purity of myoblast cell cultures we checked by Western blot analysis with anti-desmin antibodies as a myogenic marker (data not shown).

For some experiments, the lymphoblastoid cells were incubated for 24 h and 48 h in the presence of 10 µM lovastatin (Sigma, Taufkirchen, Germany). Under this condition, the cells are arrested in the G1 phase [33]. In order to initiate the cell cycle again, medium containing lovastatin was removed and the cells were then incubated in a medium containing 2 mM mevalonic acid. For inhibition of protein synthesis, lymphoblastoid cells were incubated for 24 h in the presence of 50 µg/ml cycloheximide (Sigma). After the time of incubation the cells were washed in medium without cycloheximide. Cells were then processed for gel electrophoresis and immunoblotting.

Antibodies
Ascites fluids of the following primary monoclonal antibodies were used: X223 specific for the vertebrate B-type lamin B2 [76], R27 against human lamin A and C [77], V/22 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 and required for the elongation process [78] (produced in our lab) and 8WG16 which binds specifically to the CTD heptapeptide repeat when this sequence is unphosphorylated [79]. Mouse monoclonal antibodies against emerin (NCL-emerin) were purchased from medac-(Hamburg, Germany), anti desmin MA were purchased from Sigma. The monoclonal antibody P2G3 was used to detect fibrillarin [80]. Antibodies against the following antigens were used: Lamin B Receptor (LBR, guinea pig; [81]); lamin A/C (N-18, goat, were purchased from Santa Cruz Biotechnology, Santa Cruz, USA); Ribophorin II as an endoplasmic reticulum (ER) protein marker (rabbit; provided by Dr. B. Dobberstein).

Immunofluorescence microscopy
Cultured cells grown on coverslips were fixed for 10 min in -20°C methanol, transferred for 4 min to -20°C acetone and air dried. Alternatively, the cells were fixed with methanol:acetone 1:1 for 2 min at room temperature and incubated immediately with primary antibodies. Control cell culture of muscle cells were fixed for 20 min in -20°C methanol:acetone 1:1 just before incubation with the primary antibodies. Pieces of muscle were shock frozen in isopentane cooled with liquid nitrogen. 5 μm thin cryosections of muscle were fixed with -20°C acetone for 10 min and air dried.

Fixed cells and cryo-sections were incubated with the primary antibodies for 30 min at room temperature. Anti-lamin B and anti-emerin antibodies were diluted 1:200 in PBS; anti-LBR, anti RNA pol II and anti-ribophorin antibodies were respectively diluted 1:800, 1:300 and 1:500 in PBS. R27 monoclonal and N-18 polyclonal antibodies, both directed against lamin A/C were used undiluted and diluted 1:100 in PBS respectively. After washing with PBS the samples were incubated for another 30 min with the appropriate secondary antibody conjugated to Texas Red or FITC (Dianova, Hamburg, Germany diluted 1:75 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 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). Alternatively, photos were taken with a CCD Pixel Fly camera (Klughammer, Markt Indersdorf, Germany) or samples were viewed with a Leica confocal microscope (Leica Lasertechnik GmbH, Heidelberg, Germany).

Electron microscopy
Cells and muscle were fixed, embedded in Epon and processed for electron microscopy according to standard procedures (for details see [82]).

Gel electrophoresis and immunoblotting
Proteins were resolved on 12% SDS-PAGE [83]. For immunoblots, proteins were electrophoretically transferred to nitrocellulose [84]. The membrane was blocked with 10% non-fat dry milk in TBST (10 mM Tris-HCl, pH = 8, 0,15 M NaCl, 0,05% Tween-20) followed by antibody-incubation at room temperature for 1 hour with anti-emerin antibodies, or anti-fibrillarin antibodies, or anti-desmin antibodies or anti-LBR antibodies (diluted respectively 1:1000, 1:500 and 1:400 in the blocking solution). After several washes with TBST the nitrocellulose was incubated with the secondary peroxidase-coupled anti-mouse or anti-guinea pig antibodies (Dianova,

Hamburg, Germany) at a dilution of 1:10.000 in TBST with 10% dry milk followed for 1 hour at room temperature. The blots were washed again and bound antibodies were visualized using the enhanced chemical luminescence detection system (ECL; Amersham Buchler, Braunschweig, Germany).

For some experiments the nitrocellulose was stripped of bound antibodies and reprobed as described in the manufacturers protocol (Amersham Pharmacia Biotech) with X223 (1:1000 in TBST, 1 hour incubation) R27 (undiluted, 2 hours incubation) and P2G3 (1:500 in TBST, 1 hour incubation).

Authors' contributions
BR, RK, CD, IM and AE performed the data analysis, EK produced the electronic figures, JS and HR recruited the patient and collected the cells, CRM and MCD designed and supervised the study and wrote the paper.

Acknowledgements

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