Nuclear envelope proteins and chromatin arrangement: a pathogenic mechanism for laminopathies

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The involvement of the nuclear envelope in the modulation of chromatin organization is strongly suggested by the increasing number of human diseases due to mutations of nuclear envelope proteins. A common feature of these diseases, named laminopathies, is the occurrence of major chromatin defects. Laminopathies share in some instances their clinical features, but each of them is characterized by a phenotype that involves one or multiple tissues. We previously reported that cells from laminopathic patients show an altered nuclear profile, and loss or detachment of heterochromatin from the nuclear envelope. Recent evidence indicates that processing of the lamin A precursor is altered in laminopathies featuring pre-mature aging and/or lipodystrophy phenotype. In these cases, pre-lamin A is accumulated in the nucleus and heterochromatin is severely disorganized. Moreover, altered distribution and solubility properties of heterochromatin-associated proteins such as HP1 are observed. These findings indicate that defects of chromatin remodeling are involved in the cascade of epigenetic events leading to the laminopathic phenotypes. Here we report evidence indicating that pre-lamin A is mis-localized in the nuclei of Emery-Dreifuss muscular dystrophy fibroblasts, either bearing lamin A/C or emerin mutations. Abnormal pre-lamin A-containing structures are formed following treatment with a farnesyl-transferase inhibitor, a drug that causes accumulation of non-farnesylated pre-lamin A. Pre-lamin A-labeled structures co-localize with heterochromatin clumps. These data indicate that in almost all laminopathies the expression of the mutant lamin A precursor disrupts the organization of heterochromatin domains so that affected cells are unable to maintain the silenced chromatin state capable to allow/preserve terminal differentiation. Our results further show that the absence of emerin expression alters the distribution of pre-lamin A and of heterochromatin areas, suggesting a major involvement of emerin in pre-lamin A-mediated mechanisms of chromatin remodeling.

Key words: nuclear envelope, heterochromatin, laminopathy, cell differentiation, pre-lamin A, emerin, Emery-Dreifuss muscular dystrophy.

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LMNA gene mutations result in a wide variety of different disease phenotypes

Laminopathies, a family of monogenic multi-system disorders that result from autosomal dominant and autosomal recessive mutations in the *LMNA* gene on chromosome 1q21, encoding nuclear lamins A and C, include, at the moment, 16 distinct disease phenotypes (Hegele, 2005; Maraldi *et al.,* 2005). A first group of laminopathies includes those with skeletal involvement, such as autosomal dominant Emery-Dreifuss muscular dystrophy (EDMD2), autosomal recessive Emery-Dreifuss muscular dystrophy (EDMD3), limb-girdle muscular dystrophy (LGMD1B), and dilated cardiomyopathy with conduction defect (CMD1A). In a second group are included those laminopathies with neurological involvement, such as the Charcot-Marie-Tooth disease (CMT) and several complex overlapping syndromes (OLS), characterized by lipodystrophy, muscle weakness and/or dystrophy, and cardiac conduction abnormalities. A third group identifies the laminopathies characterized by simple partial lipodystrophy, such as familial partial lipodistrophy-Dunningan type (FPLD2), lipodystrophy and cranio-facial skeletal abnormalities, such as mandibuloacral dysplasia (MADA), and progeroid syndromes, that include atypical Werner syndrome (AWRN), Hutchinson-Gilford progeria syndrome (HGPS), and restrictive dermopathy (RD).

In the large majority of the cases described, *LMNA* inherited mutations are heterozygous, leading to single amino acid exchange in lamin A, and they are spread all along the gene sequence; however, mutations are more often located at a given amino acid in MADA and HGPS, or in a restricted region in FPLD2. Interestingly, some mutants, such as those causing progeria syndromes, affect multiple cell types, whilst others, such as those causing

EDMD2, EDMD3, and FPLD2 exert effects in a cell type-specific way. Because A-type lamins are expressed in nearly every differentiated cells, it has been suggested that they may play different roles in various somatic cells.

Pathogenic models for laminopathies

Different disease models can account for the astonishing variety of tissue-restricted pathologies found in laminopathies. A-type lamin structural defects may affect the stability of the lamina and/or the proper lamina assembly (Hutchison *et al.,* 2001); this could result in typical nuclear morphological abnormalities, such as indentations and blebs of the NE, thickening of the lamina, and disorganization of the chromatin arrangement. The impaired structural stability of A-type lamins could account for an increased fragility of nuclei to mechanical stress, that could result into altered nucleus-cytoskeleton interactions and increased cell fragility (Broers *et al.,* 2004). On the other hand, A-type lamins are involved in interactions with a variety of transcription factors, including pRb (Markiewicz *et al.,* 2002), germ-cell-less (GCL) (Holaska *et al.,* 2003), and SREBP-1 (Lloyd *et al.,* 2002), and with chromatin-associated proteins (reviewed by Zastrow *et al.,* 2004; Gruenbaum *et al.,* 2005), so that they may act as regulators of transcription and cell differentiation (reviewed by Maraldi *et al.,* 2005). In the heterozygous *LMNA* mutations, it has been suggested that mutant lamin A dominantly affects the structure and/or function of the wild-type protein expressed from the normal allele. This could result in either a loss-of-function phenotype or in a gain-of-function phenotype (Gotzmann and Foisner, 2005). In some cases, the dominant negative effect of mutant lamin A has been clearly demonstrated. Mutations in the *LMNA* gene that occur in HGPS (Eriksson *et al.,* 2003; De-Sandre-Giovannoli *et al.,* 2003) and in RD (Navarro et al. 2004), by activating a cryptic splicing site, lead to the accumulation of an incompletely processed, C-terminally farnesylated truncated pre-lamin A (Eriksson *et al.,* 2003; Yang *et al.,* 2005). This pre-lamin A isoform, also called progerin, is responsible of the striking chromatin defects which are found in HGPS cells (Columbaro *et al.,* 2005). These chromatin defects, including heterochromatin loss and reduced heterochromatin-associated protein levels (Goldman *et al.,* 2004; Columbaro *et al.,* 2005) can be rescued by drug treatments aimed at lowering progerin levels (Columbaro *et al.,* 2005). Accumulation of incompletely processed pre-lamin A has been also demonstrated to occur as a consequence of heterozygous single point mutations in FPLD2 (Capanni *et al.,* 2005) and MADA (Filesi *et al.,* 2005). In MADA cells, on the other hand, accumulation of unprocessed pre-lamin A results into an altered distribution of the lamin B receptor (LBR) and in the destabilization of the heterochromatin-associated proteins histone H3 methylated at lysine 9 (H3K9), and heterochromatin protein-1β (HP1-β), thus affecting the chromatin remodelling that could represent a key event in the epigenetic changes involved into premature-aging phenotype (Filesi *et al.,* 2005). In the case of FPLD2, pre-lamin A accumulation into the nucleus can result in a gain-of-function phenotype, because the mutant protein binds to the transcription factor sterol response element binding protein 1 (SREBP1) (Capanni *et al.,* 2005). The recruitment by pre-lamin A of SREBP1, that is required for adipocyte differentiation, affects adipogenesis, possibly leading to a lipodystrophic diseased phenotype.

Heterochromatin alteration in laminopathies

Abnormal chromatin organization, mainly affecting peripheral heterochromatin, occurs in genetic diseases caused by mutations in the genes coding for inner nuclear membrane-associated proteins and lamins. In Pelger-Huet anomaly (PHA), due to mutations in the LBR gene, blood granulocytes present characteristic nuclear lobulations and an abnormal chromatin organization (Hoffman *et al.,* 2002). Nuclear alterations also occur in the Greenberg dysplasia cells, due to mutation in the LBR gene (Waterham *et al.,* 2003). The X-linked Emery-Dreifuss muscular dystrophy (EDMD1) is caused by mutations in the EMD gene that encodes for emerin (Bione *et al.,* 1994) and is characterized by altered nuclear profiles and by focal loss of peripheral heterochromatin (Ognibene *et al.,* 1999). Altered pattern of heterochromatin distribution has been, so far, identified in several laminopathies, including EDMD2 (Sabatelli *et al.,* 2001), LGMD1B, FPLD (Capanni *et al.,* 2005), MAD (Filesi *et al.,* 2005) and HGPS (Goldman *et al.,* 2004). It is conceivable that mutations affecting lamin A, emerin and LBR genes result in defective interactions of the

nuclear envelope with chromatin-associated proteins, such as HP1, thus impairing the correct localization of heterochromatin at the nuclear periphery. This, in turn, might affect the silencing of genome regions required to perform a differentiation-related program of gene repression.

LBR interacts with B-type lamins, binds DNA (Duband-Goulet and Courvalin, 2000), histone H3/H4 tetramers (Polioudaki *et al.,* 2001), the chromatin-associated protein HA95 (Martins *et al.,* 2000), and the heterochromatin protein HP1, which mediates the association of heterochromatin with the nuclear envelope (Kourmouli *et al.,* 2000).

The mechanism by which heterochromatin organization is deeply altered in laminopathies has been suggested to involve interactions between lamin A and chromatin-associated proteins that play not only a structural role but exert also transcriptional control. A-type lamins, indeed, provide scaffolds for proteins that regulate gene expression, including pRb (Mancini *et al.,* 1994; Ozaki *et al.,* 1994; Markiewicz *et al.,* 2002; Vlcek *et al.,* 2001), GCL (Holaska *et al.,* 2003), the transcriptional repressors MOK2 (Dreuillet *et al.,* 2002) and BAF (Wang *et al.,* 2002), as well as chromatin remodeling complexes (Neely and Workman, 2002). Many of the gene regulators that bind to A-type lamins are transcriptional repressors; alterations in the interaction with these repressors could account for tissue-specific defects of transcriptional activity and selectively affect stem cells during the differentiation process (Gotzman and Foisner, 2005; Squarzoni *et al.,* 2005).

Interactions with chromatin involve not only mature lamin A but also lamin A precursors

An impressive amount of experimental evidence has been recently accumulated that demonstrates typical alterations in the peripheral heterochromatin pattern in the cells from patients affected by almost all the laminopathies so far characterized. A chromatin binding site is located in the tail domain of lamin C (Glass *et al.,* 1993; Taniura *et al.,* 1995), and two DNA-binding domains are found in the rod domain of lamins A and C (Stierlé *et al.,* 2003). Recently, we focused attention on the possible involvement of both mature lamin A and pre-lamin A in chromatin-regulating mechanisms. In fact, it has been demonstrated that several diseases, all affecting large scale chromatin organization, are characterized by intranuclear accumulation of pre-lamin A (Goldman *et al.,* 2004; Capanni *et al.,* 2005).

Thus, the investigation of post-translational modifications of the lamin A precursor and of its interaction with chromatin-associated proteins that could account for transient processes of chromatin relocation with respect to the lamina/nuclear envelope, is mandatory for better approaching the pathogenic mechanism of laminopathies.

Lamin A post-translational processing

Lamins A, B and C2 undergo post-translational processing. Lamin C, which lacks the carboxy-terminal CaaX motif, is not prenylated. The precursor of lamin A has a CaaX motif at the C-terminus and undergoes post-translational modifications including prenylation, methylation and proteolytic cleavages. Pre-lamin A is the 74-kDa precursor of the 72-kDa lamin A protein. Farnesylation is required for the maturation of pre-lamin A; in fact, non-prenylated CaaX box mutants of pre-lamin A are not proteolytically processed (Holtz *et al.,* 1989). Similar effects are induced by inhibitors of protein farnesylation (Dalton and Sinenski, 1995). The absence of farnesylation does in fact prevent the binding to pre-lamin A endoprotease (Kilic *et al.,* 1997). Farnesylation occurs at the cysteine residue in the conserved CaaX motif by the enzyme farnesyl transferase (Sinensky *et al.,* 1994). The aaX tripeptide is then removed by the zinc metalloproteinase ZMPSTE24; the prenylated cysteine is methylated by a isoprenyl-cysteine carboxyl methyl transferase (Bergo *et al.,* 2003) and, finally, the C-terminal tail of 15 amino acids is removed by a second proteolytic cleavage, by ZMPSTE24. The endoproteolytic trimming and the carboxyl methylation, subsequent to isoprenylation, significantly increase the hydrophobicity of the C-termini of lamin A (Maske *et al.,* 2003).

Accumulation of pre-lamin A affects heterochromatin arrangement in several laminopathies

We recently provided evidence on the mechanism that links the accumulation of unprocessed lamin A aggregates into the nucleus and the rearrangement of the condensed chromatin. In primary cultured fibroblasts from MADA patients, we found accumulation of pre-lamin A. A certain percentage of nuclei showed an altered organization of the

nuclear envelope and of the heterochromatin associated with the presence of foci containing pre-lamin A (Filesi *et al.,* 2005). In MADA cell nuclei, HP1β and H3K9 became partially soluble by Triton X-100 treatment, suggesting that the heterochromatin is partly unstructured, as confirmed by the dramatic loss of heterochromatin in these cells (Filesi *et al.,* 2005). A typical feature of MAD and HGPS nuclei, when compared to other laminopathies, was the complete absence of heterochromatin, associated with a thickening of the nuclear lamina. Another feature of MAD cells was a marked redistribution of LBR, a nuclear envelope protein that is able to interact with heterochromatin-associated proteins such as HP1 (Kourmouli *et al.,* 2000; Polioudaki *et al.,* 2001). All these findings provide a direct evidence of an altered distribution of heterochromatin-associated proteins, due to the accumulation of pre-lamin A. This suggests that lamin A plays a key role in the correct assembly and/or stability of the heterochromatin-associated complex constituted by H3K9, HP1β, and LBR.

Analogous results have been obtained in HGPS fibroblasts. An increasing accumulation of progerin, a truncated farnesylated/methylated pre-lamin A isoform, that lacks C-terminal 50 amino acids, including the endoprotease cleavage site (Eriksson *et al.,* 2003), has been found to take place, depending on the age of the patient (Columbaro *et al.,* 2005). Correspondingly, an increasing worsening of the nuclear defects has been observed; these defects included the formation of deep nuclear envelope invaginations and focal or total loss of heterochromatin. Also the labeling pattern of mono-methyl-H3K9 staining was deeply reduced, as well as that of H3K9, indicating that both facultative and constitutive heterochromatin methylation were altered in HGPS cells (Columbaro *et al.,* 2005). Also in this case, the nuclear defects appear to be related not to a loss of mature lamin A, which is only slightly reduced in older patient cells, but to the accumulation of progerin. However, accumulation of unprocessed lamin A could occur at different steps, that lead to the formation of either non-farnesylated or farnesylated pre-lamin A. At the moment, accumulation of non-farnesylated pre-lamin A has not been found in laminopathic disorders.

However, the interplay of pre-lamin A with heterochromatin constituents can be experimentally analyzed by investigating the effects of the accumulation of non-farnesylated or farnesylated pre-lamin A on chromatin organization by utilizing drugs affecting lamin A processing and/or chromatin organization. Farnesyltransferase inhibitors have been extensively used to induce the accumulation of non-farnesylated pre-lamin A. These drugs induce accumulation of non-farnesylated pre-lamin A because they impair subsequent processing of the precursor protein by the endoprotease ZMPSTE24 (Sasseville and Raymond, 1995). We obtained evidence that the treatment of C2C12 myoblasts with a farnesyltransferase inhibitor caused accumulation of pre-lamin A at the nuclear periphery, associated with the formation of nuclear lamina invaginations protruding into the nucleoplasm. Moreover, condensed heterochromatin areas were increased in the nuclei accumulating pre-lamin A, as revealed by DAPI staining and electron microscope observations (Maraldi *et al.,* 2005). The farnesyltransferase inhibitor FTI-277, that does not affect farnesylation of lamin B or ras proteins (Adjei *et al.,* 2000), but selectively impairs pre-lamin A farnesylation, induces deep nuclear lamina invaginations in human fibroblasts. Nuclei also present large heterochromatin aggregates at the nuclear interior (Figure 1). The accumulation of farnesylated pre-lamin A, induced by the inhibitor of ZMPSTE24 Opa, on the contrary, resulted in the appearance of enlarged misshapen nuclei, in which pre-lamin accumulation was restricted at the nuclear rim (Lattanzi *et al.,* in preparation). These results suggest that all the events that impair lamin A processing result in alteration of the nuclear shape (either invaginations of the lamina or enlargement and blebs).

Distinct classes of laminopathies

In laminopathies affecting muscle, defective lamin phosphorylation (Ellis *et al.,* 1998; Cenni *et al.,* 2005), nuclear envelope defects (Gilchrist *et al.,* 2004; Broers *et al.,* 2005) and focal loss of peripheral heterochromatin (Ognibene *et al.,* 1999; Sabatelli *et al.,* 2001) are common features independent of the site at which the mutation occurs. Most EDMD and CMD mutations are distributed in the rod domain of lamin A/C, while almost all mutations causing FPLD and progeroid laminopathies are due to mutations of the lamin A/C C-terminus. While lamin A precursor is not

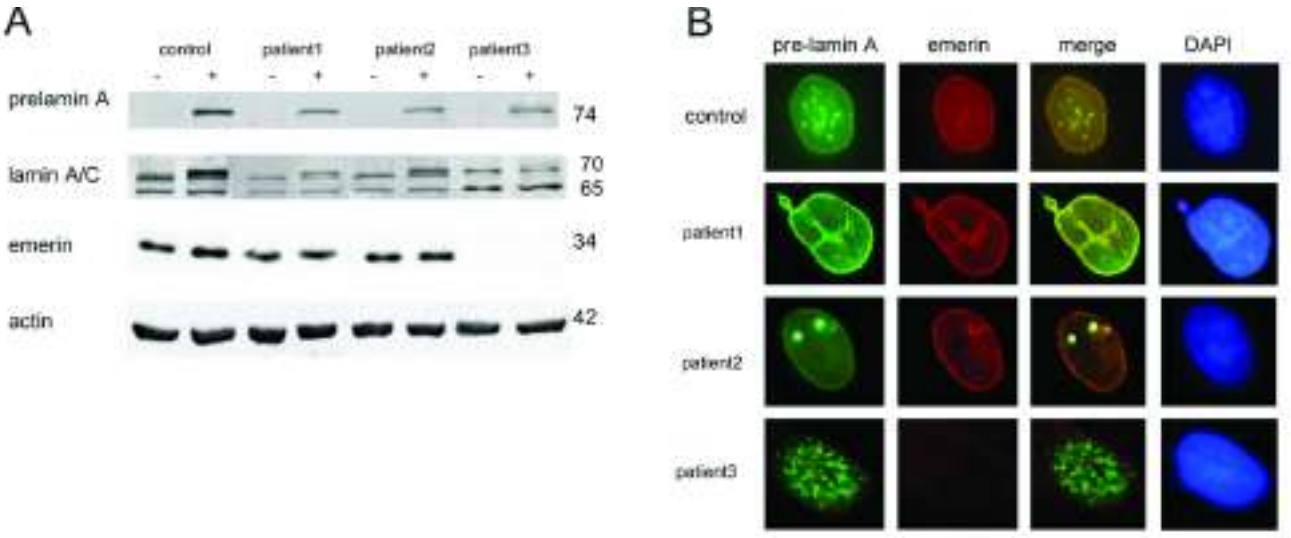

Figure 1. Pre-lamin A expression and localization are altered in EDMD fibroblasts. Fibroblast cultures were obtained from patient biopsies following informed consent. The following mutations were diagnosed: patient 1, *LMNA* R401C; patient 2, *LMNA* R249Q, R190Q; patient 3, EMD, null mutation (C to T mutation at nucleotide 1740,which causes a codon 228>stop). (A) Western blot analysis of pre-lamin A, lamin A and lamin C in control, EDMD2 and EDMD1 fibroblasts. Cultured fibroblasts at passage 6 were tretaed with FTI-277 (20 μM for 18 hours), then harvested and lysed in buffer and subjected to 5-20% gradient SDS-PAGE and Western blot analysis with anti-pre-lamin A (Santa Cruz, SC-6214), anti-lamin A/C, anti-emerin and anti-actin antibody (actin labeling shows equal loading amount). Molecular weight markers are in kDa. (B) Double immunofluorescence labeling of pre-lamin A and emerin in control, EDMD2 and EDMD1 fibroblasts following FTI-277 treatment. Anti-pre-lamin A polyclonal antibody is detected by anti-pre-lamin A (Santa Cruz, SC-6214) and revealed by FITC-conjugated anti-goat IgG, emerin is detected by anti-emerin monoclonal antibody and revealed by Cy3-conjugated anti-mouse IgG. DAPI staining of chromatin is shown.

accumulated in EDMD and CMD (Capanni *et al.,* 2005 and our unpublished results), in the other well defined group of laminopathies, including FPLD, MAD, RD and progeroid syndromes, accumulation of unprocessed lamin A occurs (Capanni *et al.,* 2005; Filesi *et al.,* 2005; Navarro *et al.,* 2005; Liu *et al.,* 2005; Goldman *et al.,* 2004; Glynn and Glover, 2005; Scaffidi and Misteli, 2005). In this second class of laminopathies, characterized by progeroid and/or lipodystrophy phenotype, nuclear defects consist of gross nuclear shape abnormalities, thickening of the nuclear lamina and severe heterochromatin defects (Capanni *et al.,* 2003; Filesi *et al.,* 2005; Liu et al. 2005), appearing as complete heterochromatin loss in a percentage of MAD (Filesi *et al.,* 2005) and HGPS cells (Goldman *et al.,* 2004), or as large heterochromatin clumps at the nuclear periphery of RD cells (Shackleton *et al.,* 2005).

Analogous loss of heterochromatin, associated with deep nuclear envelope invaginations occur in HGPS fibroblasts, due to the accumulation of progerin, a truncated farnesylated/methylated pre-lamin A isoform. Also in this case, reduction of H3K9 indicated an altered mechanism of heterochromatization in the presence of uncleavable pre-lamin A (Scaffidi and Misteli, 2005). Whilst the overexpression of wild-type lamin A did not rescue nuclear alterations (Scaffidi and Misteli, 2005), this was obtained by correction of the altered *LMNA* splicing (Scaffidi and Misteli, 2005) or by the combined use of drugs that reduce progerin level and modify the chromatin arrangement, such as mevinolin and trichostatin A (Columbaro *et al.,* 2005). Removal of progerin by the combined action of these drugs also restored the ribonucleoprotein distribution, suggesting the obtainment of a functional recovery. Recovery of the nuclear defects have been also reported by using FTIs in HGPS cells (Mallampalli *et al.,* 2005; Toth *et al.,* 2005). These results demonstrate that nuclear defects, and mainly the heterochromatin loss in progeric laminopathies are due to a dominant negative effect of accumulating pre-lamin A and not to a loss of functional mature lamin A.

Mis-localization of pre-lamin A in EDMD

Besides the autosomal form of Emery-Dreifuss muscular dystrophy (EDMD2), caused by *LMNA* mutations spread all along the gene sequence, the X-linked form (EDMD1) is due to mutations of the

EMD gene on the Xq28 locus, that codes for the inner nuclear membrane-associated protein emerin (Bione et al. 1994). The clinical phenotypes display a wide overlap, suggesting a common pathogenic mechanism for EDMD1 and EDMD2. Most *EMD* mutations result in a null phenotype and emerin is not detected in cell lysates. Similarities are also found in the cellular defects, consisting in an altered organization of the nuclear lamina and in the detachment of heterochromatin or focal heterochromatin loss at the nuclear periphery. Since recent evidence indicates that the processing mechanism of the lamin A precursor plays a key role in the pathogenesis of several laminopathies, we investigated expression and localization of the pre-lamin A in EDMD1 and EDMD2 fibroblasts in the attempt to find out a correlation between the heterochromatin defects observd in EDMD and possible alterations of pre-lamin A processing. Fibroblasts from four EDMD2 and two EDMD1 patients were examined in this study. The corresponding mutations are reported in the legend to figure 1, where representative results obtained in cell cultures from three different patient biopsies are shown. Western blot analysis demonstrates that EDMD2 fibroblasts express a slightly reduced amount of lamin A/C with respect to control fibroblasts (Figure 1A). The amount of lamin A precursor in EDMD2 cellular lysates is consistently reduced to undetectable levels (Figure 1A). Interestingly, both lamin A/C and pre-lamin A amount are also reduced in EDMD1 cells bearing null emerin mutations (Figure 1A). In EDMD1 and EDMD2 cells subjected to FTI-277 treatment, pre-lamin A level is increased, but it appears lower than that detected in FTI-treated controls (Figure 1A). Immunofluorescence analysis of pre-lamin A in FTI-277-treated fibroblasts gave unexpected results. Though pre-lamin A was undetectable by immunofluorescence labeling in untreated EDMD cells (*not shown*), following FTI-277 treatment the specific anti-lamin A precursor antibody labeled the nuclear envelope in each patient cell line (Figure 1B). In about ten per cent of EDMD1 and EDMD2 nuclei, pre-lamin A-labeled intranuclear aggregates were observed (Figure 1B). These structures were not detected in control nuclei (Figure 1B). It is noteworthy that the shape of pre-lamin A-labeled aggregates varied from one cell line to the other. In patient 1 and 2, the pre-lamin A-containing structures co-localized with DAPI-stained heterochromatin areas (Figure 1B), while in cells from the other EDMD2 patient appeared as micronuclei (Figure 1B). The pre-lamin A-labeled aggregates observed in EDMD1 emerin-null cells co-localized with brigtly fluorescent chromatin foci (Figure 1B). These data show for the first time that the localization of the lamin A precursor protein is altered in EDMD cells and that anomalous pre-lamin A-labeled aggregates may contribute to segregate heterochromatin areas. Moreover, the unexpected alteration of pre-lamin A distribution observed in emerin-null EDMD1 fibroblasts, suggests a link between pre-lamin A and emerin affecting nuclear organization. The observation that different *LMNA* mutations elicit different effects on the distribution of pre-lamin A, strongly suggests that more than one binding partner may regulate the proper targeting of the lamin A precursor in fibroblast nuclei. Different mutations may well interfere with one or the other pre-lamin A inter-molecular interaction, thus leading to defective organization of the nuclear lamina. It is noteworthy that pre-lamin A is rapidly processed in the cell, so that defects here observed may correspond to an altered mechanism affecting transient processes of pre-lamin A targeting, in turn interfering with chromatin organization. A recent report (Hubner *et al.,* 2006) shows that mutated pre-lamin A bearing EDMD mutations forms aggregates in transfected HeLa cells and these aggregates segregate lamin A-binding proteins SREBP1 and pRB. Further studies will evaluated the presence of these proteins in pre-lamin A –containing structures found in EDMD fibroblasts. Moreover, a major goal of our study, will be the definition of the effect of LMNA mutations on pre-lamin A targeting in myoblasts, which represent the affected cell type in EDMD.

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