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# Proteasome-mediated degradation of integral inner nuclear membrane protein emerin in fibroblasts lacking A-type lamins

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**Abstract**

We previously identified and characterized a homozygous *LMNA* nonsense mutation leading to the absence of A-type lamins in a premature neonate who died at birth. We show here that the absence of A-type lamins is due to degradation of the aberrant mRNA transcript with a premature termination codon. In cultured fibroblasts from the subject with the homozygous *LMNA* nonsense mutation, there was a decreased steady-state expression of the integral inner nuclear membrane proteins emerin and nesprin-1α associated with their mislocalization to the bulk endoplasmic reticulum and a hyperphosphorylation of emerin. To determine if decreased emerin expression occurred post-translationally, we treated cells with a selective proteasome inhibitor and observed an increase in expression. Our results show that mislocalization of integral inner nuclear membrane proteins to the endoplasmic reticulum in human cells lacking A-type lamins leads to their degradation and provides the first evidence that their degradation is mediated by the proteasome.


*Keywords:* Nuclear envelope; Lamin; Emerin; Nesprin; Proteasome; Muscular dystrophy

Lamins are intermediate filament proteins that form the nuclear lamina, a fibrous meshwork underlying the inner nuclear membrane of higher eukaryotic cells [1]. In human, two types of lamins are expressed in somatic cells according to their sequences and biochemical properties, A-type and B-type. Lamins A and C, encoded by the *LMNA* gene, are the predominant A-type lamins in mammalian somatic cells and arise from alternative RNA splicing. Two genes, *LMNB1* and *LMNB2*, respectively, encode the somatic mammalian B-type lamins B1 and B2 [1]. While lamins B1 and B2 are expressed in most of all somatic cells, the expression of A-type lamins is developmentally regulated and generally restricted to differentiated cells [2,3]. The nuclear lamina is attached to the inner nuclear membrane via interactions with integral proteins, among them emerin, nesprin-1α, LBR, and LAPs [4]. Lamins A and C also interact with chromatin [5] and DNA [6]. The nuclear lamina functions in maintenance of nuclear architecture, DNA replication, and the synthesis of mRNA [4].

Mutations in *LMNA* cause several human diseases affecting different tissues, which are sometimes referred to as "laminopathies" [4]. One of these diseases is limb-girdle muscular dystrophy type1B (LGMD1B), an autosomal dominant disorder characterized by symmetrical weakness starting in the proximal lower limb muscles and gradually affecting the upper limb muscles [7,8]. It is associated with cardiac conduction disturbances and dilated cardiomyopathy, similar to autosomal dominant Emery–Dreifuss muscular dystrophy and isolated cardiomyopathy with

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conduction defect 1A, which are also caused by *LMNA* mutations.

The molecular mechanisms underlying the various disease phenotypes caused by *LMNA* mutations are unknown. However, several investigators in the field have proposed two main hypotheses to explain the tissue-specific effects observed in laminopathies [4]. The ''mechanical stress'' hypothesis proposes that abnormalities in the nuclear lamins caused by *LMNA* mutations lead to structural defects and increased nuclear fragility, eventually resulting in nuclear disruption in mechanically stressed cells. The ''gene expression'' hypothesis proposes a tissue-specific role of lamins in transcription and abnormalities in expression of genes involved in cell differentiation or maintenance of the differentiated state of certain tissues if there are *LMNA* mutations. These two hypotheses are not necessarily mutually exclusive, as, for example, defective nuclear mechanics have been linked to altered expression of stress-activated genes in fibroblasts lacking A-type lamins [9].

We previously investigated the molecular and cellular alterations in cultured skin fibroblasts from two members of a family with LGMD1B carrying a *LMNA* nonsense mutation (Y259X) [10]. A heterozygous 66-year-old woman had a typical LGMD1B phenotype, whereas her grandchild carrying two mutated *LMNA* alleles, leading to absence of A-type lamins, died at birth after preterm delivery by cesarean section [11]. Fibroblasts from the homozygous neonate had nuclei with marked structural defects and mislocalization of emerin and nesprin-1α to the endoplasmic reticulum [10]. In this report, we further examine cells from the subject with the homozygous *LMNA* Y259X mutation and present evidence that emerin ''escaped'' from the inner nuclear membrane to the bulk endoplasmic reticulum in these cells lacking A-type lamins undergoes proteasome-mediated degradation.

## Materials and methods

*Reagents.* Lactacystine and cycloheximide (CHX) were purchased from Sigma–Aldrich. Lactacystine was prepared as a 10 mM stock solution in dimethyl sulfoxide and stored at −80 °C until use. CHX was dissolved in ethanol as a stock solution of 100 μg/ml.

*Cells.* Skin fibroblasts were obtained from a male child homozygous for the Y259X *LMNA* mutation who died at birth after delivery at 30 gestational weeks [10,11]. Fibroblasts from a 12-week-old fetus with no *LMNA* mutation were used as control. Fibroblasts were obtained from skin biopsies obtained after informed consent of the parents and complied with ethical guidelines approved by the Institutional Review Board. Fibroblasts were maintained at 37 °C in a humidified incubator containing 5% $CO_2$ in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf bovine serum (Invitrogen) and 0.1% gentamycin.

*RNA extraction.* At 80% confluence, media were removed from cultures and cells immediately lysed by addition of 2 ml RNAplus (Bioprobes) directly to culture plate. Total RNA was prepared as described by the manufacturer. Adequacy and integrity of extracted RNA were assessed by Agilent analysis (Agilent Technologies) and concentrations measured by ultraviolet absorbance spectroscopy.

*Indirect immunofluorescence microscopy.* Fibroblasts were grown on coverslips and washed with phosphate-buffered saline (PBS) then fixed as previously described [10,12]. Fibroblasts were then incubated with the primary antibodies for 1 h at room temperature. Primary antibodies used were anti-lamin A/C monoclonal MANLAC (1:10, provided by G.E. Morris), anti-lamin B1 polyclonal (1:250, a gift from J.C. Courvalin), anti-emerin polyclonal (1:100, provided by G. Morris), and anti-nesprin-1α (1:200, provided by E.M. McNally). After washes with PBS, fibroblasts were incubated with secondary antibodies (1:500). Secondary antibodies used were Alexa fluor 568 goat anti-mouse immunoglobulin G (Molecular Probes) and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Chemicon). Cells were then washed with PBS and slides mounted in Mowiol (Santa Cruz Biotechnologies) with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (dapi). Immunofluorescence microscopy was performed using an Axiophot microscope (Carl Zeiss). Micrographs were processed using Adobe Photoshop 5.5 (Adobe Systems).

*Real-time reverse transcription-polymerase chain reaction (RT-PCR).* Reverse transcription was performed on 5 μg total RNA using the SuperSript First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed using a LightCycler (Roche Diagnostics). PCR mix (20 μl) included 2 μl of each diluted reverse transcription product, 4 mmol/l $MgCl_2$, and 300 nmol/l of each primer in 1× LightCycler DNA Master SYBR Green. Specific primers for cDNAs were chosen with the LightCycler program according to following Genbank Accession Nos.: *LMNA* (Accession No. NM_170707), *LMNB1* (Accession No. NM_005573), *EMD* (Accession No. NM_000117), *SYNE1* (Accession No. NM_022027), *LBR* (Accession No. NM_002296), and *NUP153* (Accession No. NM_005124). For *LMNA*, the primers were common to both lamin A and lamin C mRNA transcripts. PCR was performed using the following conditions: denaturation and enzyme activation at 95 °C/15 min and cycling at 95 °C/30 s, 64 °C/10 s, and 72 °C/10 s. Analysis was carried out with the LightCycler 3.5 software (Roche). Relative levels of mRNA expression were calculated according to the $\Delta\Delta C_T$ method [13]. Individual expression values were normalized by comparison with β-actin mRNA.

*Western blot analysis.* Fibroblasts ($3 \times 10^6$) were harvested from each culture at the appropriate time interval, washed with ice-cold PBS, and total protein extracted as previously described [10,12]. For Western blot analysis, equal amounts of protein (10 μg) were electrophoresed on 10% SDS–polyacrylamide gels, transferred to nitrocellulose membranes (Invitrogen) by electroblotting at 4 °C for 1 h at 400 mA, and stained with Ponceau S. Primary antibodies used were anti-lamin A/C Jol5 monoclonal antibody (1:250, provided by provided by C. Hutchison), anti-lamin B1 (1:2000, provided by J.C. Courvalin), anti-emerin (1:500, Novocastra), anti-nesprin-1α (1:500, provided by E.M. McNally), and anti-β-actin polyclonal antibody (1:2000, Sigma). Secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit antibodies (1:2000, DAKO). Protein detection was performed using a SuperSignal West Pico Chemiluminescent Substrate (Pierce) and visualized using Hyperfilm ECL (Amersham).

## Results and discussion

### *Expression of mRNAs encoding nuclear envelope proteins in cells homozygous for the LMNA Y259X mutation*

Our initial aim was to determine whether the presence of homozygous Y259X *LMNA* nonsense mutation affects the expression of mRNA encoding lamins A and C in cultured skin fibroblasts. Total RNA was extracted from skin fibroblasts cultured from unaffected fetal tissue and from the subject with the homozygous mutation (fibroblasts$^{Y259X/Y259X}$). Although similar amounts of total mRNA were analyzed as revealed by β-actin mRNA detection, real-time quantitative RT-PCR revealed a significant decrease of the two different lamin A/C mRNA transcripts encoded by *LMNA* in fibroblasts$^{Y259X/Y259X}$ compared to controls (Fig. 1A). Expression of mRNAs encoded by *LMNB1*