Repetitive disruptions of the nuclear envelope invoke temporary loss of cellular compartmentalization in laminopathies

Winnok H. De Vos 1,5, Frederik Houben 1,†, Miriam Kamps 1, Ashraf Malhas 3, Fons Verheyen 1,2, Juliën Cox 1, Erik M.M. Manders 5,7, Valerie L.R.M. Verstraeten 3, Maurice A.M. van Steensel 3, Carlo L.M. Marcelis 8, Arthur van den Wijngaard 4, David J. Vaux 6, Frans C.S. Ramaekers 1 and Jos L.V. Broers 1,*

1Department of Molecular Cell Biology, CARIM-School for Cardiovascular Diseases, 2Electron Microscopy Unit, Department of Molecular Cell Biology, 3Department of Dermatology, GROW—School for Oncology and Developmental Biology and 4Department of Clinical Genetics, CARIM-School for Cardiovascular Diseases, Maastricht University Medical Center, PO Box 616, NL-6200 MD Maastricht, The Netherlands, 5Department of Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Ghent, Belgium, 6Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, UK, 7Centre for Advanced Microscopy, Section of Molecular Cytology, University of Amsterdam, Kruislaan 316, NL-1098 SM Amsterdam, The Netherlands and 8Department of Clinical Genetics, Radboud University Nijmegen Medical Centre, The Netherlands

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The nuclear lamina provides structural support to the nucleus and has a central role in nuclear organization and gene regulation. Defects in its constituents, the lamins, lead to a class of genetic diseases collectively referred to as laminopathies. Using live cell imaging, we observed the occurrence of intermittent, non-lethal ruptures of the nuclear envelope in dermal fibroblast cultures of patients with different mutations of lamin A/C. These ruptures, which were absent in normal fibroblasts, could be mimicked by selective knockdown as well as knockout of LMNA and were accompanied by the loss of cellular compartmentalization. This was demonstrated by the influx of cytoplasmic transcription factor RelA and regulatory protein Cyclin B1 into the nucleus, and efflux of nuclear transcription factor OCT1 and nuclear structures containing the promyelocytic leukemia (PML) tumour suppressor protein to the cytoplasm. While recovery of enhanced yellow fluorescent protein-tagged nuclear localization signal in the nucleus demonstrated restoration of nuclear membrane integrity, part of the mobile PML structures became permanently translocated to the cytoplasm. These satellite PML structures were devoid of the typical PML body components, such as DAXX, SP100 or SUMO1. Our data suggest that nuclear rupture and loss of compartmentalization may add to cellular dysfunction and disease development in various laminopathies.

INTRODUCTION

The nuclear envelope is the principal barrier dictating bidirectional communication between the nucleus and cytoplasm of the cell (1). Directly underlying the double lipid bilayer is an intimately connected meshwork of intermediate filament proteins, the nuclear lamina, which provides structural support to the nucleus and has a central role in defining nuclear organization (2). Defects in one of its major constituents, the A-type nuclear lamins, lead to a spectrum of diseases collectively referred to as laminopathies. Disease manifestations range from systemic premature aging, such as observed...
in Hutchinson–Gilford Progeria syndrome (HGPS), to muscular dystrophies and lipodystrophies (3). One hypothesis for explaining disease development premises that gene regulation becomes affected due to disturbed interactions between mutant A-type lamins and chromatin or transcription factors (4). Another frequently postulated hypothesis argues that mutations in the LMNA gene cause mechanical weakening of the lamina and nuclear envelope, leading to accumulation of nuclear damage under physical stress conditions (5,6). While both theories are supported by experimental data, so far no conclusive mechanism for the development of laminopathies has been established.

It is widely accepted that the nuclear membrane becomes dramatically affected in laminopathy cells; dilation of the perinuclear space (7), aggregation of nuclear pore complexes (8) and even complete local absence of an overt lamina (9,10) have been documented both in cell cultures and in patient tissues. However, little is known about the implications of these structural alterations of the nuclear envelope for nuclear-cytoplasmic communication. While it has been shown that nuclear transport becomes altered in cell lines overexpressing specific mutant (pre-)lamin A isoforms (11), there is no clear view on the general impact of LMNA mutations on cellular compartmenatalization. Here, we demonstrate that different mutations of A-type lamins cause intermittent, non-lethal ruptures of the nuclear envelope, which lead to temporary exchange of proteins between nucleus and cytoplasm, as well as permanent translocation of nuclear protein complexes to the cytoplasm and vice versa. The potential impact of this loss of cellular compartmenatalization on disease development is discussed.

RESULTS

70 kDa dextran particles accumulate in the nucleus of laminopathy cells

Passage-matched primary fibroblasts (all between passage 10 and 15), derived from different laminopathy patients, were scrape loaded with Texas Red-labeled dextran (MW > 70 kDa), which should normally not pass the nuclear pores of an intact nuclear envelope (Fig. 1a). Using confocal microscopy, we assessed cellular dextran distribution at different time points after loading cells of a progeroid syndrome patient, carrying a compound heterozygous p.M540T and p.T528M missense mutation on different alleles of the LMNA gene (M540T/T528M), as well as in cells from a patient with a lethal nonsense Y259X homozygous mutation in LMNA (Y259X/Y259X). For comparison, we used passage-matched, scrape loaded dermal fibroblast cells (NHDFα, +/+ ) from a healthy donor as a control. Immediately after scrape loading, no living (attached) control cells containing cytoplasmic dextran could be detected with intranuclear dextran. During the first 24 h after scrape loading, laminopathy cells showed a significantly and progressively higher nuclear uptake of fluorescent dextran molecules compared with control cells (Fig. 1b). After 24 h, only 4.6 ± 0.6% of the control +/+ cells showed nuclear staining, while 21.4 ± 2% of M540T/T528M cells (p = 7.4E−11) and 22.2 ± 0.8% of Y259X/Y259X cells (p = 2.3E−10) were detected with positive nuclear staining. Hence, nuclear permeability was significantly higher in these cells than could be anticipated from basal dextran uptake, for instance due to mitotic events, as seen in control cells.

Reversible loss of NLS-EYFP from laminopathy nuclei

The number of dextran-positive nuclei in laminopathy cells increased gradually with time after scrape loading (Fig. 1B). To determine whether this increase in nuclear permeability was due to reduced pore function leading to gradual, continuous uptake or rather due to larger structural defects resulting in more abrupt leakage of the nuclear membrane, we performed detailed live-cell imaging experiments using enhanced yellow fluorescent protein-tagged nuclear localization signal (EYFP-NLS). We chose this marker for nuclear compartmenatalization to guarantee minimal interference with basal cell physiology. Dermal fibroblast cultures from patients suffering
from various laminopathies were transfected with EYFP-NLS and monitored in time using confocal time-lapse microscopy. Both short-term (2 h at 1 min intervals) and long-term (24 h at 5–15 min intervals) recordings were acquired. All interphase cells from two different batches of NHDF control cells retained a stable intranuclear EYFP-NLS signal intensity throughout a 2–24 h recording period (Table 1). In contrast, several laminopathy-patient fibroblasts showed a sudden decrease in EYFP-NLS signal in the nucleus and concurrent increase in EYFP-NLS signal in the surrounding cytoplasm (Fig. 2, Table 1, Supplementary Material, Fig. S1 and Videos S1–S3). NPC-poor regions were also devoid of lamin B1 (Supplementary Material, Fig. S3).

Table 1. Comparison of nuclear abnormalities and EYFP-NLS visualized nuclear ruptures in different laminopathy fibroblast cultures

<table>
<thead>
<tr>
<th>Human dermal fibroblast culture</th>
<th>Gene</th>
<th>Mutation</th>
<th>Cells (% ± SD) with nuclear abnormalities (herniations and/or honeycombs, n = 3 × 100 cells)</th>
<th>EYFP-NLS expressing cells (%) showing nuclear ruptures in a 2 h recording period (no. of rupturing cells/total no. of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHDFo-1</td>
<td>LMNA</td>
<td>+/+</td>
<td>5.7 ± 0.6</td>
<td>0 (0/23)</td>
</tr>
<tr>
<td>NHDFo-2</td>
<td>LMNA</td>
<td>+/+</td>
<td>2.3 ± 1.5</td>
<td>0 (0/149)</td>
</tr>
<tr>
<td>Double-null</td>
<td>LMNA</td>
<td>Y259X/Y259X</td>
<td>63.0 ± 5.0 (P = 3.9E−05)⁴</td>
<td>49.5 (93/188)</td>
</tr>
<tr>
<td>Prageroid</td>
<td>LMNA</td>
<td>M540T/T528M</td>
<td>38.8 ± 9.0 (P = 7.3E−04)⁴</td>
<td>29.1 (39/134)</td>
</tr>
<tr>
<td>Father progeroid</td>
<td>LMNA</td>
<td>+/T528M</td>
<td>13.5 ± 11.6 (P = 0.29)</td>
<td>14.3 (3/21)</td>
</tr>
<tr>
<td>Mother progeroid</td>
<td>LMNA</td>
<td>+/M540T</td>
<td>9 ± 4.6 (P = 0.31)</td>
<td>0 (0/23)</td>
</tr>
<tr>
<td>HGPS</td>
<td>LMNA</td>
<td>+/G608G</td>
<td>13.0 ± 2.3 (P = 7.7E−04)⁴</td>
<td>13.6 (21/154)</td>
</tr>
<tr>
<td>FPLD</td>
<td>LMNA</td>
<td>+/R439C</td>
<td>16.5 ± 3.5 (P = 3.6E−03)⁴</td>
<td>24.6 (15/61)</td>
</tr>
<tr>
<td>RD</td>
<td>ZMPSTE24</td>
<td>c.1085-1086insT/c.1085-1086insT</td>
<td>27.6 ± 3.2 (P = 8.6E−05)⁴</td>
<td>5.2 (3/58)</td>
</tr>
<tr>
<td>HCM</td>
<td>LMNA</td>
<td>+/R644C</td>
<td>5.0 ± 1.7 (P = 0.56)</td>
<td>7.7 (7/91)</td>
</tr>
</tbody>
</table>

⁴Significantly different from control NHDFo-1 fibroblast culture as determined from a Students’ t-test (P-value < 0.05).

Nuclear ruptures are a direct consequence of lamin A/C abnormalities

In general, nuclear rupture was most frequently detected in laminopathy cells with the most severe phenotype (Table 1). For instance, cells devoid of any functional lamin A/C showed rupture events in almost 50% of all cells (49.5%). A direct correlation between the absence of normal, functional A-type lamins and the frequency of ruptures was found by comparing the cells from a M540T/T528M patient to cells from the patients’ parents. While 29.1% of the patient cells showed ruptures, fibroblasts from the father, who carries the p.T528M mutation, displayed less ruptures (14.3%, +/T528M) and cells from the mother, carrying the p.M540T mutation, did not show any ruptures at all (+/M540T).

To confirm that reduction in functional lamin A/C proteins was a causal factor for nuclear membrane rupture, a stable 3T3 lamin A/C knockdown fibroblast cell line (12) and embryonic fibroblasts (MEF) from lamin A/C knockout mice (Lmna−/−) were transfected with EYFP-NLS and compared with their respective wild-type counterparts. 18.5% (5/27) of the 3T3 knockdown cells and up to 48% (11/23) of the Lmna−/− MEFs showed ruptures in a 120 min recording period, whereas none was observed in wild-type 3T3 cells (0/40) or wild-type MEFs (0/21).

Strikingly, the rupture phenomenon was also observed, albeit to a lesser extent, in laminopathy cells associated with the accumulation of unprocessed, or partly processed mutant prelamin A. 13.6% of the EYFP-NLS-positive cells from a HGPS (+/G608G) patient showed ruptures, while 5.2% of the cells from a patient suffering from a restrictive dermopathy (RD), with homozygous c.1085-1086insT truncating mutations in the ZMPSTE24 gene, ruptured in a 2 h recording period. The occurrence of nuclear ruptures in aforementioned cells made us wonder whether the phenomenon was not only caused by a reduction in mature lamin A, but could also be invoked by the accumulation of prelamin A intermediates, such as progerin. Indeed, introduction of EGFP–D50–lamin A in normal human dermal fibroblasts (NHDF) cells resulted in nuclear rupture...
events in 19.6% of all recordings (11/56), suggesting the involvement of multiple causative factors.

Repetitive nuclear rupture does not impede cell division

Upon prolonged imaging of the most severely affected cell lines, i.e. Y259X/Y259X and M540T/T528M, we observed several intermittent rupture events within the same cell occurring at intervals ranging from 5 min up to several hours (Fig. 2). Even after multiple nuclear ruptures, we observed successful cell division in these long-term recordings (Supplementary Material, Videos S3 and S4). The duration of mitosis in cells that ruptured was 30–60 min, similar to that observed in normal NHDFα fibroblasts.

Nuclear rupture is accompanied by transient, bidirectional translocation of regulatory proteins

To test whether the observed rupture events affected nucleocytoplasmic compartmentalization of essential regulatory proteins, we visualized transcription factors that should normally be restricted to the cytoplasm or the nucleus and are specifically involved with cellular stress responses. As a cytoplasmic marker we used RelA, a component of the dimeric NF-κB complex, which remains sequestered in the cytoplasm in the absence of stress stimuli (13). RelA was found to enter the nucleus of double-transfected Y259X/Y259X cells as well as Lmna<sup>−/−</sup> MEFs (Fig. 3A). Simultaneous exit of mCherry-NLS confirmed that this was due to nuclear rupture and not to a sudden stress or stimulus event.

Cyclin B1, a pivotal cell-cycle regulator normally restricted to the cytoplasm of interphase cells (14), also entered the nucleus during rupture (Supplementary Material, Fig. S4), confirming that nuclear ruptures are accompanied by an influx of various cytoplasmic components.

To study the behaviour of intranuclear components, we transfected Y259X/Y259X cells with a GFP-fusion construct expressing octamer binding transcription factor 1 (OCT1) which has been shown to reside in an insoluble nuclear fraction (15), and is partly sequestered at the

Figure 2. Non-lethal nuclear ruptures. (A) Selected images from a time-lapse recording of an M540T/T528M fibroblast transfected with EYFP-NLS, sampled at 2 min intervals for 2 h (Supplementary Material, Video S1). Note the nuclear herniation (arrowhead) that forms the initiation site for rupture: the herniation gradually grows until it bursts, accompanied by a direct decrease in intranuclear EYFP signal and increase in cytoplasmic EYFP signal. Subsequently, a gradual recovery of the nuclear signal can be observed along with a reformation of the herniation. A second burst is again followed by a recovery of the nuclear signal indicating restoration of the nuclear membrane integrity. (B) Temporal quantification of nuclear and cytoplasmic EYFP-NLS signal (mean intensity) of the cell shown in (A); (C) montage of a confocal time-lapse recording of a Y259X/Y259X cell 24 h after transfection with EYFP-NLS, sampled at 30 min intervals (see also Supplementary Material, Video S3). Confocal image stacks have been projected according to the maximum pixel intensity and rigidly registered (correlation-based translation and rotation) for display purposes. The cell undergoes three consecutive rupture events (indicated by orange arrowheads), visible by the simultaneous decrease in nuclear signal and increase in cytoplasmic signal, each time followed by a restoration of the nuclear signal. After ≈17 h of monitoring, the cell goes through mitosis; (D) montage of a confocal time-lapse recording of a Y259X/Y259X cell 24 h after double transfection with EGFP–NUP153 and mCherry-NLS, sampled at 5 min intervals, showing a rupture initiated at a site with reduced abundance of nuclear pore complexes (top left, see also Supplementary Material, Video S5). The scale bars represent 10 μm.
nuclear periphery (16). Indeed, we found that nuclear rup-
tures caused part of the OCT1 to translocate to the cyto-
plasm. Efflux of OCT1 was far subtler than for
EYFP-NLS and appeared to be limited to focal or diffuse
regions in the nucleus (Fig. 4A, Supplementary Material,
Fig. S5 and Videos S6 and S7).

Impaired compartmentalization may affect downstream
gene expression

We hypothesized that the impairment in spatiotemporal
nucleocytoplasmic compartmentalization in laminopathy
cells could have functional consequences for cellular func-
tion. For example, OCT1 regulates genes that are essential
for the cellular stress response (17), which could mean that
failure to compartmentalize OCT1 correctly causes
dysregulation of these genes. As the downstream genes
have been characterized in the mouse and the pathological
cells of human origin could be biased by different genetic
backgrounds, we determined the expression levels of
OCT1-responsive genes in 3T3 Lmna knockdown cells and
Lmna<sup>−/−</sup> MEFs with respect to their wild-type controls and
compared these with published values for Oct1<sup>−/−</sup> mouse
cells (17). Out of nine genes that were investigated, the ex-
pression of four genes (Serping1, Rdml1, IL6 and Gas5) was
significantly altered in the same direction for the Lmna
knockdown, the Lmna<sup>−/−</sup> and the Oct1<sup>−/−</sup> cells. For two
more genes (Mmp13 and Gpx3), there were statistically sig-
nificant changes in the same direction for the Lmna<sup>−/−</sup> and
the Oct1<sup>−/−</sup> cells, with no significant changes in the Lmna
knockdown cells from wild-type controls, consistent with a
lamin A/C-dependent effect (Fig. 4B).

Figure 3. Nuclear ruptures lead to influx of cytoplasmic components. (A) Montage of a confocal time-lapse recording of a MEF Lmna<sup>−/−</sup> cell 24 h after double
transfection with EGFP-RelA and mCherry-NLS, demonstrating transient influx of RelA during nuclear rupture (at time point 00:05). Scale bar represents
10 μm. (B–D) Electron microscopy images of Y259X/Y259X cells showing the presence of different particles, including microfilaments (B, arrows), vesicles
(C, D, E, arrows), mitochondria (C, E, arrowheads) and myeloid bodies (D, open arrows).
Nuclear rupture is accompanied by permanent translocation of macromolecular complexes

Triggered by the observations of uncoordinated translocation of regulatory proteins, we wondered whether larger gene-regulatory protein complexes could become relocalized during rupture as well. EYFP-tagged promyelocytic leukaemia (PML) proteins were selected as markers, as these assemble into multi-protein complexes termed PML nuclear bodies, which have an important role in transcription control and DNA repair and are in general confined to the nuclear compartment (18). Indeed, upon nuclear rupture (indicated by efflux of co-transfected EYFP-NLS), part of the EYFP-tagged PML bodies translocated from the nucleus to the cytoplasm (Fig. 5A, Supplementary Material, Video S8). As the nuclear membrane integrity recovered (indicated by restoration of nuclear EYFP-NLS signal), translocated PML structures remained excluded from the apparently restored nucleus. Some PML structures that remained in the cytoplasm gradually fragmented into smaller structures that sometimes even dissolved completely. The number of PML structures that translocated during an individual rupture ranged between 0 and 5, but due to the repetitive nature of this event as much as 24% of the original number of nuclear PML structures was found to move out of the nucleus in long-term recordings (Supplementary Material, Video S8). These findings were confirmed by immunocytochemical staining of endogenous PML bodies and high-content cytometry, thereby excluding any artifacts that could arise from transfection. Only 3.2 ± 1.3% of NHDFa control cells (n = 300) demonstrated cytoplasmic PML body staining, while 59.6 ± 2.1% of Y259X/Y259X cells (n = 300) showed the presence of intensely staining PML bodies in the cytoplasm (Fig. 5B). All other patient cell lines examined also demonstrated a higher number of cells with cytoplasmic PML bodies, ranging from 5 to 18% (data not shown). Interestingly, cytoplasmic PML structures never contained other typical PML body components such as DAXX or SP100 and were often also devoid of SUMO1. This was demonstrated by immunostaining of non-transfected cells (Fig. 5B) as well as immunolocalization after live imaging of PML structure translocation upon rupture (Supplementary Material, Fig. S6).

Figure 4. Nuclear rupture leads to efflux of transcription factor OCT1 and dysregulation of Oct1-responsive genes. (A) Maximum intensity projections of a confocal time-lapse recording acquired at 5 min intervals of a Y259X/Y259X patient cell 24 h after transfection with GFP-OCT1, showing temporary and localized loss of nuclear GFP-OCT1 during nuclear rupture at different time points (indicated by orange arrowheads, see also Supplementary Material, Video S6). Contours were drawn to mark the nuclear boundary, the dotted line in the first frame demarcates the cell boundary and the inset shows a contrast-stretched, magnified view of the rectangular selection indicated in the first frame, to demonstrate the subtle increase in cytoplasmic GFP-OCT1 signal during rupture. (B) Fold changes in Oct1-responsive gene expression as measured by comparative real-time polymerase chain reaction (PCR) in Lmna knockdown (kd) 3T3 cells and Lmna−/− MEFs with respect to their wild-type controls. Error bars represent 95% confidence intervals and significant log-fold changes (P < 0.05) are indicated by a star. The table compares log-fold ratio values with those described for an Oct1−/− MEF cell line (17).
Similarly, translocation of large components from the cytoplasmic side to the nucleus could be indicated using electron microscopy of Y259X/Y259X cells. Prominent inclusions of non-nuclear components were found in the nucleus. These inclusions fully resemble subcellular structures normally present in the cytoplasm (Fig. 3B), such as microfilaments, vesicles, mitochondria and myeloid bodies. The inclusions are not surrounded by an additional nuclear membrane, indicating that they are not the result of nuclear membrane folding or intranuclear tubular invaginations.

DISCUSSION

It is well established that defective production or aberrant post-translational processing of A-type lamins elicits a broad spectrum of laminopathies that include tissue-specific (adipose, muscle, peripheral nerve) as well as systemic disorders. Considering the large variety of disease manifestations, several non-exclusive hypotheses have been proposed to relate the pathophysiology of laminopathies to lamin functions (2). These are either based on the involvement of lamins in maintaining mechanical integrity of the nucleus (structural hypothesis), their role in modulating transcription and signaling pathways by serving as docking sites for regulatory proteins (gene expression hypothesis) or the toxicity of accumulated pre-lamin A isoforms (toxicity hypothesis). However, current hypotheses put less focus on the primary function of the nuclear envelope in basal cell physiology, namely the maintenance of a highly selective barrier between nucleus and cytoplasm. Yet, several lines of evidence implicate an important role to distorted nucleocytoplasmic transport in cellular degeneration. For instance, nuclear import is reduced in cell lines overexpressing mutant lamin A intermediates (11), as well as in cells undergoing senescence (19). In addition, in aging cells, impaired nuclear pore function has been observed, leading to increased nuclear permeability and the leakage of cytoplasmic proteins, such as tubulin, into the nucleus (20). The link between the influx of cytoplasmic tubulin into the nucleus with the aging process as well as neurodegenerative diseases (21) emphasizes the role of (deregulated) nuclear transport in development of human pathologies. Our findings on the occurrence of nuclear ruptures in cells from different laminopathy patients clearly support this connection, but

Figure 5. Nuclear rupture leads to translocation of mobile PML structures. (A) Montage of selected time points of a confocal time-lapse recording of an Y259X/Y259X cell 24 h after cotransfection with EYFP-NLS and EYFP-PML, sampled at 3 min intervals. Confocal image stacks have been projected according to the maximum pixel intensity and rigidly registered (correlation-based translation and rotation) for display purposes. At time point 02 h 03 min, a nuclear rupture event takes place (evident by the sudden drop in diffuse nuclear signal) leading to the immediate translocation of two PML nuclear bodies (bottom, yellow and red arrowhead) and, again, escape of two PML nuclear bodies 30 min later at time point 02 h 33 min (top, cyan and magenta arrowhead). While the nuclear signal gradually restores, the cytoplasmic PML bodies translocate further away from their initial escape sites and fragment into smaller particles (cf. magenta and cyan arrowhead at time point 03 h 33 min). (B) Immunofluorescence staining of +/+ and Y259X/Y259X cells shows that cytoplasmic PML structures are present in the laminopathy cells and do not contain typical PML body components, such as DAXX, SUMO or SP100. Scale bars represent 10 μm.
also add a novel aspect to the suggested paradigm: instead of a gradual, chronic transport problem, suggested to be caused by decreased levels of import factors (19) or the presence of oxidatively damaged pore complexes (20), we now document sudden disruptions of the nuclear compartment which lead to an immediate intermingling of nuclear and cytoplasmic components. Although the exact nature of these ruptures is not yet known, there appears to be a correlation between the reduction of mature, non-mutant lamin A/C proteins and the frequency of nuclear ruptures. Different lines of evidence point to this correlation. First, both Lmna knockdown in 3T3 cells and Lmna knockout in MEFs result in nuclear ruptures, which were never observed in wild-type cells. Secondly, the progeroid patient cells (M540T/T528M), expressing only mutant A-type lamins, show a much higher number of nuclear ruptures, compared to the cells from the heterozygous parents, each bearing one normal LMNA allele. While lamin A/C deficiency has been shown to result in increased nuclear fragility and apoptosis in cells exposed to mechanical stress (22), the ruptures described here occur under basal culturing conditions without application of external force are transient in nature and are non-lethal. Ruptures often initiated at weak spots of the nucleus, typified by reduced presence or even complete absence of other nuclear envelope components, such as lamin B or nuclear pore complexes, indicating a structural basis for this phenomenon. Indeed, structural alterations in the lamina either weaken or stiffen the nuclear envelope, in case of a lamin A/C deficiency (23) or progerin accumulation (24), respectively. Both conditions may render the nucleus more susceptible to local damage. In HGPS cells, a reduced compliance of rigid nuclei to pulling forces, exerted by the cytoskeleton, could cause local fractures in the nuclear envelope (25). Alternatively, in lamin A-deficient cells, a defective connection with the cytoskeleton (26) augments nuclear mobility and deformation (23), thereby possibly increasing the risk of ruptures, especially at fragile sites of the imperfect nuclear envelope.

The exact impact of dynamic nuclear ruptures on cell behavior, differentiation and cellular lifespan remains elusive, but it is conceivable that the abrupt loss of compartmentalization affects cellular functioning in several ways. For instance, the sudden mislocalization of essential transcription factors upon nuclear rupture may alter gene expression. Previous studies have indicated that lamins form a scaffold for tethering several signaling proteins and transcription factors (ERK, pRb, SREBP1, OCT-1 etc.) and thereby modulate their activity (27,28). As we show here, LMNA mutations will not only alter these interactions directly but also invoke unintentional, bidirectional diffusion of various transcription factors or signaling proteins; a phenomenon, which may be missed by classical biochemical approaches. The altered stoichiometry of resident nuclear proteins is prone to shift DNA-binding kinetics and thereby indirectly affect gene regulation permanently or temporarily (29–31). This is supported by our data on Oct1-responsive gene expression, which indeed becomes altered in LMNA+/− cells in a similar way as in Oct1−/− cells and in an opposite sense with respect to Lmnb+/−/− cells (16), plausibly due to reduced abundance of nucleoplasmic Oct1. The fact that not all of the investigated Oct1-responsive genes react similarly to the (partial) loss of Lmna transcripts may be explained by the fact that ruptures invoke translocation of a variety of transcription factors (e.g. ReA) and thus influence gene expression in a highly complex manner. Moreover, A-type lamins influence gene expression in a multifactorial fashion through additional mechanisms such as sequestration of transcription factors at the periphery and by their role in chromatin organization (27,28).

The alterations in Oct1-responsive gene expression may cause cells failing to act timely and adequately to oxidative stress. As such, it raises the intriguing possibility that nuclear ruptures connect structural and gene-regulatory aspects of disease development. Alternatively, mislocalization of cellular proteins could impose an extra load on the cell for recuperating material to the correct compartment by means of nucleocytoplasmic transport. This process may become especially strenuous in a context of aging or pre-lamin A accumulation, i.e. conditions where reduced nuclear import is observed (11,19,20,32). Accumulation of translocated material in the wrong cellular compartment may be targeted for degradation by the proteasomes, but large amounts of mislocalized protein could increase the risk of proteasome saturation and consequent aggresome formation or even induction of apoptosis (33).

Next to transient shuttling of regulatory proteins, ruptures were also accompanied by the translocation of macromolecular complexes, such as PML containing structures. These mobile PML structures were devoid of typical PML body components DAXX and SP-100 and often also SUMO1. In composition, they resemble PML microstructures, which are produced after heavy metal or heat-shock treatment (34), but unlike these PML microstructures, the satellite PML structures we observed can be as large as classical nuclear PML bodies. Two possible translocation mechanisms can be envisaged: either satellite PML structures exist in the nucleus that lack interactions with chromatin rendering them more mobile and allowing them to escape the nucleus upon rupture; or alternatively, translocated PML bodies rapidly lose their other components, for instance due to desumoylation [cf. mitotic PML accumulations (35)], leading to gradual defragmentation. Although it is tempting to speculate on a role in stress response, analogous to PML microstructures (34), the exact function of these satellite PML structures remains to be resolved.

Dynamic ruptures of nuclear envelope herniations have previously been observed in HIV-infected cells (36). It is now known that HIV and also other virus infections weaken the nuclear lamina, potentially to facilitate viral entry into the nucleus, confirming the critical role for lamins in maintaining subcellular compartmentalization. Moreover, strong indications exist that nuclear rupture is not confined to in vitro cell systems. Electron microscopic studies show local absence of nuclear membranes in cardiomyocytes of heart biopsies (9,10), as well as in striated muscle cells (37) of laminopathy patients. As various other pathologic cell cultures such as small lung carcinoma cells also display a dramatic decrease in lamin A/C expression (38), we speculate that temporary decompartmentalization may have a more generic relevance for development of diseases associated with an altered lamin A/C metabolism.
MATERIALS AND METHODS

Cell culture

The following human dermal fibroblast cultures from healthy volunteers and patients were used in this study: (i) two different batches of NHDF (NHDFα, +/+ , Promocell, Heidelberg, Germany); (ii) cells from a patient with a lethal laminopathy phenotype due to a nonsense Y259X homozygous mutation in the LMNA gene (double-null, Y259X/Y259X) (26); (iii) HGPS cells (+/G608G) (39); (iv) cells from a compound progeroid syndrome patient, carrying a LMNA M540T and a LMNA T528M mutation on different alleles (M540T/T528M) as well as cells from the patients’ father and mother, who have no disease symptoms (39). The apparently healthy father carries the c.1583C>T (p.T528M) mutation in exon 9 (+/T528M). This mutation is associated in the literature with familial partial lipodystrophy (FPLD) (40). The mother carries the c.1619T>C (p.M540T) mutation in exon 10 and is also symptom-free (+/M540T); (v) Cells from a patient with FPLD carrying a R439C mutation in the LMNA gene (+/R439C) (41); (vi) cells from a patient suffering from RD, with homozygous c.1085_1086insT truncating mutations in the ZMPSTE24 gene. This frameshift mutation leads to a premature termination codon of the protein and inactivation of the ZMPSTE24 enzyme, resulting in prelamin A accumulation in patient nuclei (42); (vii) cells from a recently diagnosed patient with hypertrophic cardiomyopathy (HCM) with a defect in lamin A only (+/R644C). Informed consent for inclusion in this study was obtained from the patients or from their parents. In addition, NIH-3T3 mouse fibroblasts, wild-type cells and cells with stable lamin A/C knock-down (12) as well as wild-type and lamin A/C knockout MEFs (Lmna−/−) were used (43). Cells were cultured in advanced DMEM/F12 medium (Gibco, Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin/glutamin, at 37°C and 5% CO2, according to standard procedures. In case of direct comparison, passage-matched cells were used.

Transfection

The following constructs were used for transfection: EYFP-NLS or mCherry-NLS (44) (generous gifts from Dr J. Goedhart, University of Amsterdam, the Netherlands), EGFP-LMN1B, GFP-OCT1 (16), EGFP–NUP153 (45) (a kind gift of Birthe Fahrenkrog, ULB, Brussels, Belgium), GFP-RelA (Addgene plasmid 23255,46) and/or with EGFP-PML (47) (a kind gift from Dr J. Wiegant, Leiden University, the Netherlands), Cyclin B1-GFP (14,48) (kindly provided by Dr J. Pines, Cambridge, UK) and EGFP-D50-lamin A (Addgene plasmid 17653,49). For transfection, GeneJammer (Stratagene, La Jolla, CA, USA) or Lipofectamine 2000 (Invitrogen) was used, according to the manufacturer’s instructions.

Live-cell imaging

Time-lapse recordings were made 24–48 h after transfection as described previously (12,50). Care was taken to only select cells with moderate expression levels and correct (initial) localization patterns. At least three different biological replicates were used. Cellular condition was also verified by phase contrast microscopy, to assure that cells showed a normal morphology without excessive vacuole formation. Live-cell imaging was performed on a Nikon A1R confocal microscope, mounted on a Nikon Ti body, equipped with a Perfect Focus System and a microscope incubator equilibrated at 36.5°C. Recordings were made using a 60×/1.4 Plan Apo oil immersion lens. Alternatively, live-cell imaging was performed on an inverted fluorescence microscope (Leica DIRBE, Leica Microsystems BV, Rijswijk, The Netherlands), equipped with a black and white CCD camera (CA4742-95, Hamamatsu), a polychrome II polychromator as light source for fluorescence (TILL Photonics, Martinsried, Germany) and a 20 × 0.7 Plan Apo lens. Image acquisition was achieved using proprietary software, while image processing, analysis and annotation were performed in ImageJ freeware v1.43 (51), Adobe Photoshop® 9.0.2 and Adobe Illustrator® 12.0.1 (Adobe Systems Inc., CA, USA). Time-lapse experiments were performed with cells ranging from passages 20 to 23.

Immunofluorescence labeling

Human fibroblasts were grown, fixed and immunostained as previously described (12) using the following antibodies: PML (PG-M3) mouse monoclonal antibody (1:1000, Sc-966, Santa Cruz, Heidelberg, Germany); lamin A/C mouse monoclonal antibody Jol2 (1:50, kindly provided by Prof. C. Hutchinson, Durham, UK); lamin B rabbit polyclonal antibody (1:500, ab16048, Abcam, Cambridge, UK); lamin B (C-20) goat polyclonal antibody (1:500, sc-6216, Santa Cruz); MAB414 mouse monoclonal antibody to nuclear pore complex components (1:2000, ab24609, Abcam), DAXX rabbit polyclonal antibody (1:200, HPA008736, Sigma-Aldrich, St Louis, MO, USA), SP100 rabbit polyclonal antibody (1:200, HPA016707, Sigma-Aldrich) and SUMO1 rabbit polyclonal antibody (1:200, ab32058, Abcam). As secondary antibodies, FITC-conjugated goat anti-mouse Ig (1:50, SBA/ITK Birmingham, AL, USA), Texas Red conjugated goat anti-rabbit Ig (1:50, SBA/ITK), Dylight 488 donkey anti-mouse IgG (1:300, 715-486-150 Jackson ImmunoResearch Laboratories, PA, USA), Dylight 549 donkey anti-rabbit IgG (1:300, 711-506-152 Jackson ImmunoResearch Laboratories) and Dylight 649 donkey anti-goat IgG (1:300, 705-506-150 Jackson ImmunoResearch Laboratories) were used. Immunostained cells were mounted in Vectashield (Invitrogen) containing 1 ng/ml 4′,6-diamino-2-phenylindole (DAPI) and visualized using a Leica TCS SPE confocal laser scanning fluorescence microscope (Leica DMRBE, Mannheim, Germany) using LAS-AF software (version 2.2, Leica) or with a Nikon TE2000 Eclipse widefield microscope.

Dextran scrape loading

Passage-matched cells (between passages 10 and 15) were treated using a variation of the scrape loading technique (52). Cells, grown on coverslips to near confluency, were submerged in culture medium containing 1 mg/ml Dextran-Texas Red (70 kDa, Molecular Probes, Oregon). Using a blunted hypodermic needle, the glass surface was scratched and...
wounded cells were allowed to take up dextran for 5 min. While severely damaged cells at the central region of the wound area in general detached, at least 300 viable, attached cells containing dextran could be visualized using this technique and were scored on a 25 mm round coverslip. For each experiment, three coverslips were used per time point, and all experiments were repeated at least three times. Next, cells were washed three times with the medium and fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) after variable incubation periods (0, 4, 8 and 24 h). Nuclei were counterstained with DAPI and the presence of intranuclear dextran was assessed by analyzing confocal z-series.

Image analysis

For scoring of nuclear shape aberrations or cytoplasmic PML bodies, either a high-content analysis was performed as described before (53) or scored manually. Nuclear aberrations were identified as honeycomb structures after lamin labeling or nuclear herniations and holes after DAPI staining. In scoring of PML bodies, a second non-specific channel was acquired (550LP) to prevent autofluorescent foci from skewing the analysis. Mitotic and early G1 cells, which may exhibit some cytoplasmic PML bodies, were rejected from the analysis based on intensity and shape criteria of the DAPI-staining.

Electron microscopy

LMNA double-null cells (Y259X/Y259X) were cultured for at least 48 h in a culture dish after which the medium was discarded. Next, the cells were shortly rinsed with PBS and subsequently fixed at room temperature with 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) supplemented with 0.5 mM CaCl2 for at least 24 h. The cells were then postfixed with 2% OsO4 in cacodylate buffer (pH 7.4) containing 1.5% potassium ferricyanide at 4°C for at least 48 h in a culture dish after which the medium was discarded. Next, the cells were shortly rinsed with PBS and subsequently fixed at room temperature with 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) supplemented with 0.5 mM CaCl2 for at least 24 h. The cells were then postfixed with 2% OsO4 in cacodylate buffer (pH 7.4) containing 1.5% potassium ferricyanide at 4°C for 1 h. After a short rinse in cacodylate buffer, the cells were further dehydrated in graded ethanol series before embedding in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM100 electron microscope at 80 kV.

Real-time PCR

Wild-type and Lmna−/− MEFs or wild-type and Lmna knockdown 3T3 cells were seeded at equal densities and harvested the day after, at ~75% confluency for RNA extraction. RNA was extracted from five biological replicates for each cell type, using High Pure RNA Isolation Kit (Roche, West Sussex, UK), according to the manufacturer’s instructions. Superscript III reverse transcriptase (Invitrogen, Paisley, UK) was used for first-strand cDNA synthesis. Real-time polymerase chain reaction (PCR) was performed using an Applied Biosystems Step One Plus apparatus and the FAST SYBR GREEN MasterMix (Applied Biosystems, CA, USA). Relative gene expression values were determined using the 2−ΔΔCt method (54), using the housekeeping gene β-actin (Actb, forward primer: ACTTCTATGTTGTTGACGAG, reverse primer: CCAGATTTCTTCCATCATGCTG) as an internal standard and Lmna (forward: GCAACAGTCTCAATAGGACCA, reverse: GTCCAGATTACATGTAGC) as an internal control. The following primer pairs were used for Oct1-responsive target genes: Prdx2 (forward: TGGGCTTGTAGTGCTAC, reverse: CGTCTGTAAGCG AGGCTCT), Gas5 (forward: CGCCTGTTATCCAGTTCC, reverse: AGCTGTGCGGCATTCTGAG), Sod1 (forward: TAC TGATGGAGCTGGAACCC, reverse: GAACCATCCACTTG AGCA), Gpx3 (forward: GCAACAGTCTCAATAGGACCA, reverse: GTCCAGATTACATGTAGC), Serping1 (forward: AAGTTGGTGTCTTGGGAACA, reverse: GCCCAATTCGATGACCATA), Mmp13 (forward: GTGCTTTGAGGTGTAC CAGA, reverse: TGATGAAACCTGGAAACAAGCA), It6 (forward: AAGTCCGGAGAGACTTC, reverse: CAAGATGCGAAGCTTTC, reverse: GGAAGGGGAGTCAAGTAAGC), Rdm1 (forward: TGCCCTCTGTATCCAGTCC, reverse: CAAGAGCAACCTTCACTGGT).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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