LUMA interacts with emerin and influences its distribution at the inner nuclear membrane

Luiza Bengtsson* and Henning Otto‡
Freie Universität Berlin, Institut für Chemie und Biochemie, Thielallee 63, 14195 Berlin, Germany
*Present address: MDC-FMP, Robert-Rössle-Str. 10, 13125 Berlin, Germany
†Author for correspondence (e-mail: h.otto@biochemie.fu-berlin.de)

Accepted 19 November 2007
Journal of Cell Science 121, 536–548 Published by The Company of Biologists 2008
doi:10.1242/jcs.019281

Summary
We present here a first characterization of LUMA, an unique integral inner nuclear membrane (INM) protein. LUMA is a highly conserved protein even in some bacteria and shares a PFAM domain of unknown function with orthologs from many species. Assessing LUMA topology by using protease protection of membrane-inserted LUMA and antibody epitope accessibility assays reveals that LUMA contains four transmembrane domains and a large hydrophilic domain located between membrane spans 1 and 2. The large hydrophilic domain is exposed to the perinuclear space whereas both LUMA termini reside cyto- or nucleoplasmically. Nuclear envelope targeting of LUMA mainly depends on the membrane spans. LUMA's transmembrane domains also promote homooligomerization. LUMA binds A- and B-type lamins and depends on A-type lamins for its INM localization. Furthermore, it interacts with emerin. Both downregulation of LUMA and overexpression of dominant-negative acting LUMA fragments causes redistribution of emerin. We propose that LUMA functions as a tetraspanin-like membrane organizer and has the potential to contribute to the pathomechanism of dystrophic diseases, such as Emery-Dreifuss muscular dystrophy.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/4/536/DC1

Key words: Emerin, Emery-Dreifuss muscular dystrophy, Lamins, LUMA, Nuclear envelope

Introduction
The nuclear envelope (NE) defines the border of the nucleus: an inner nuclear membrane (INM) and an outer nuclear membrane (ONM) join at the nuclear pores, which harbor the nuclear pore complexes (NPCs) that guard the entry to and exit from the nucleus. Although the ONM is continuous with the rough endoplasmic reticulum (rER), both ONM and INM have specific sets of integral membrane proteins (Schirmer and Foisner, 2007). There are ~80 known INM proteins (Dreger et al., 2001; Schirmer et al., 2003; Schirmer and Foisner, 2007). These enrich at the nucleoplasmic face of the nuclear membrane by binding lamina and chromatin components in the nucleus (Gruenbaum et al., 2005). The nuclear lamina, an extremely stable filament meshwork consisting of intermediate filament proteins, the lamins, supports the INM. Two types of lamins – type A and B – build the lamina. The lamin-network is thought to act both as tensegrity element (Hutchison, 2002) and as a scaffold, organizing the spatial arrangement of proteins required for cellular functions, such as DNA replication, gene expression, chromosome attachment, signaling and nuclear positioning (Gruenbaum et al., 2005; Schirmer and Foisner, 2007).

The best studied INM proteins belong to the family of LEM-domain proteins, which were named for a domain present in LAP2, emerin and MAN1. The LEM-domain is the binding site for the small chromatin protein barrier-to-autointegration factor (BAF) (Margalist et al., 2007) and thus provides a link between the nuclear membrane and chromatin. A similar role is postulated for the lamin B receptor, which binds histones H3 and H4, and heterochromatin protein HP1 (Ye et al., 1997; Polioudaki et al., 2001). Besides BAF, LEM-domain proteins also bind transcriptional co-repressors (e.g. Btf, GCL) (Holaska et al., 2003; Haraguchi et al., 2004; Melcon et al., 2006) and transcription factors (SMADs, E2F) (Bengtsson, 2007). Additionally, some recruit chromatin-modifying complexes (Somech et al., 2005) and some can influence transcription directly (Nili et al., 2001). Thus, INM proteins have active roles in the regulation of gene expression.

The importance of the NE in the regulation of cellular processes has been highlighted by the fact that more than 200 mutations in lamins and lamina-associated proteins cause 18 different diseases collectively called laminopathies (Broers et al., 2006; Rankin and Ellard, 2006). Laminopathies range from Emery-Dreifuss muscular dystrophy (EDMD) to Hutchinson-Gilford progeria syndrome (Rankin and Ellard, 2006). Most laminopathies are linked to mutations in A-type lamins, for example the autosomal-dominant EDMD (Bonne et al., 1999). Interestingly, mutations in the lamina-associated LEM-domain protein emerin cause a X-chromosome-linked recessive form of EDMD (Bione et al., 1994). In cells of most patients suffering from any of the EDMD forms, emerin is missing from the NE (Bengtsson and Wilson, 2004). The exact molecular mechanism leading to disease is not yet known. However, cumulating evidence from biochemical and cell biological data suggests that protein-protein interactions important for structural stability of the nucleus and for tissue-specific gene expression are disturbed by laminopathic mutations (Burke and Stewart, 2002; Broers et al., 2006).

Despite recent progress in identifying and characterizing proteins that build the NE, its organization and its molecular interactions are not very well understood. We previously identified NE proteins isolated from neuroblastoma cells using a proteomics approach, and
LUMA at the inner nuclear membrane

revealed two novel proteins of the INM: the mouse ortholog of Unc-84, now called Sun1 (Haque et al., 2006), and LUMA (Dreger et al., 2001). An independent proteomics screen confirmed the presence of LUMA at the nuclear membrane (Schirmer et al., 2003). Here we present the first characterization of LUMA. We show that LUMA is unique among the characterized INM proteins in several aspects: (1) LUMA is a highly conserved protein and present in most sequenced species, including bacteria, (2) LUMA oligomerizes through its transmembrane domains and, (3) most of the LUMA sequence is located in the ER lumen. Furthermore, LUMA interacts with emerin and participates in controlling its distribution along the nuclear membrane. Thus, LUMA appears to be an integral membrane protein that spatially and functionally organizes protein complexes of the INM and, therefore, has the potential to cause pathological changes of the NE.

Results

LUMA is highly conserved and widely distributed among species and tissues

BLAST analysis starting with murine LUMA [UniGene (NCBI) Cluster Mm.38801] returns orthologous sequences from all sequenced vertebrate and insect genomes, many unicellular eukaryotes (e.g. Paramecium tetraurelia, Tetrahymena thermophila), some plants [Arabidopsis thaliana, Phaeodactylum tricornutum (algae), Solanum tuberosum (potato)] and several bacteria (e.g. Mesorhizobium loti, Syntrophobacter fumaroxidans, Silicibacter sp. TM1040, see Fig. 1A for representative examples). So far, we never found more than one ortholog per species. Alignment of the orthologous sequences highlights LUMA as a highly conserved protein (supplementary material Figs S1, S2). Human LUMA (TMEM43, Unigene Cluster Hs.517817) is 93% identical to murine LUMA. Other orthologs, including the bacterial ones, are typically 30% identical and 50% similar to murine LUMA (Fig. 1A). All orthologs, except those from plants, share the topology of predicted transmembrane domains (Fig. 1B) and a PFAM Domain of Unknown Function, DUF1625 (Fig. 1B) (Bateman et al., 2004), which comprises about two-thirds of murine LUMA (residues 121-373). This domain has so far only been found in LUMA orthologs. Thus LUMA is a unique protein; no other characterized nuclear membrane protein is that highly conserved. The high degree of sequence conservation as well as the spread of LUMA throughout the species predicts an essential, non-redundant function for LUMA in the cell.

UniGene data suggest that LUMA mRNA is present in a wide variety of human tissues. We therefore tested mRNA levels by RT-PCR and found that LUMA expression levels vary strongly between tissues (Fig. 1C). The highest amounts of LUMA mRNA are detectable in the placenta, whereas most other tissues show low to very low LUMA expression. Notably, LUMA mRNA was undetectable in the skeletal muscle.

LUMA is an ER protein enriched at the inner nuclear membrane

We have previously shown that LUMA localizes to the NE when transiently expressed at moderate levels in COS-7 cells, as indicated by immunofluorescence staining of the nuclear rim (Dreger et al., 2001). Others have shown the same localization of GFP-tagged LUMA overexpressed in BHK cells (Goodchild and Dauer, 2005). To characterize endogenous LUMA, we raised a polyclonal antibody against the hydrophilic domain of LUMA (residues 53-309; for a characterization of the antibody see supplementary material Fig. S5). We used the affinity-purified anti-LUMA antibody to examine the localization of endogenous LUMA in HeLa, NIH 3T3 (Fig. 2A), 293T and undifferentiated C2C12 cells (not shown). LUMA localizes to the nuclear rim in all cell lines tested, which is consistent with the localization of V5-tagged LUMA transiently expressed in COS-7 cells (Dreger et al., 2001) and of GFP-tagged LUMA in BHK cells (Goodchild and Dauer, 2005). Furthermore, the nuclear-rim staining of LUMA overlaps with the staining for emerin (Fig. 2A), LAP2 and SUN1 (not shown), and surrounds the nuclear lamin A/C (Fig. 2A). The fraction of LUMA that is found outside the nuclear membrane presumably resides in the non-nuclear domains of the ER (Fig. 2A, arrows). Thus, LUMA is an integral membrane protein of the ER and appears to be specifically retained at the INM.

Since the rim-staining alone is not sufficient to distinguish between INM and ONM localization, we performed several biochemical and cell biological assays to check whether LUMA behaves like a typical INM protein. For example, several INM
proteins are mislocalized to the non-nuclear domains of the ER when lamins are missing (Östlund et al., 2006). We examined whether this is the case for LUMA by staining mouse embryonic fibroblasts (MEFs) that lack A-type lamins (Sullivan et al., 1999) with LUMA-specific antibody (Fig. 2B). We found that LUMA accumulated at the NE of wild-type MEFs (LMNA+/+) but not in LMNA–/– MEFs. The lack of LUMA-signal at the NE in LMNA–/– MEFs was not a result of degradation of LUMA: equal amounts of the protein are present in LMNA–/– and wild-type LMNA+/+ MEFs (Fig. 2C). Thus, like other INM-proteins, LUMA requires A-type lamins for its retention at the NE.

To examine the biochemical properties of LUMA, we probed fractionated cellular extracts from N2a cells separated on SDS-PAGE and transferred to nitrocellulose with the affinity-purified anti-LUMA antibody. The antibody recognized a single band at 45 kDa (Fig. 2F). As a result of our fractionation scheme (Fig. 2E), we expected any INM protein or INM-protein interactor to specifically enrich in the NE, but not in the ER fraction. This is the case for emerin, LAP2, lamin A/C and also LUMA (Fig. 2F). Emerin has recently been described to possess ER-specific functions (Salpingidou et al., 2007), thus we were not surprised to find a substantial amount of emerin in the ER fraction (Fig. 2F).

Likewise, LUMA is present in the ER fraction, reflecting the immunofluorescence ER-staining seen by the anti-LUMA serum (Fig. 2A, arrows). As ER marker proteins we employed calnexin (total ER) and Sec61 (rER) (Gorlich and Rapoport, 1993; Greenfield and High, 1999). Since the ONM is part of the rER, Sec61 reflects the pattern of emerin and LUMA throughout the fractionation. Calnexin is distributed throughout the ER and, therefore, shows a reduced relative amount in the nuclear fraction.
due to the loss of ER membranes as a result of the nuclear preparation. Its relative protein amount increases again in NE, because the NE preparation results in strongly reduced protein complexity of the sample (Fig. 2F, equal amounts of protein were loaded on the gel). Thus, the subcellular localization of LUMA closely mirrors that of the ER/INM protein emerin.

Lamins and most lamin-binding INM proteins resist extraction with detergents unless solubilized at a high ionic strength (e.g., 1 M NaCl) (Foisner and Gerace, 1993). To determine whether LUMA behaves similarly, we treated whole cells, isolated nuclei and isolated nuclear envelopes with Triton X-100 in the presence of either 0.1 M or 2 M NaCl (Fig. 2G). Interestingly, most of the LUMA was extractable from whole cells already in low-salt conditions. This fraction was smaller in isolated nuclei and almost disappeared in purified nuclear envelopes. Vice versa – compared with whole-cell extracts – the fraction of LUMA that resisted extraction at 2 M NaCl is increased in purified nuclear envelopes extracts. Thus, although there appears to be a considerable pool of LUMA that is not anchored at the INM, LUMA behaves at the NE like a typical lamina and/or chromatin-binding protein of the INM.

To independently show a tight association of LUMA with nuclear components like lamin and/or chromatin, we extracted parental and LUMA-overexpressing HeLa cells with 0.5% Triton X-100 before fixation and double-stained the cells for LUMA and emerin (Fig. 2D). The pre-extraction removed most of the ER LUMA-signal, but left the rim-staining intact (compare Fig. 2A with 2D). We thus conclude that LUMA tightly interacts with lamin and/or chromatin and is therefore an ER protein specifically enriched at the INM.

The large hydrophilic LUMA domain resides in the ER lumen

The mouse gene for LUMA is located on chromosome 6 (M. musculus 1200015A22Rik), the human LUMA gene on chromosome 3 (H. sapiens MGC3222); both consist of 12 predicted exons. LUMA mRNA (mouse, gi:12836214) encodes a protein of 400 amino-acid residues with a predicted mass of 44.8 kDa, which is consistent with its apparent molecular mass of 45 kDa in SDS-PAGE (Fig. 2F). The Kyte-Doolittle hydropathy profile (Kyte and Doolittle, 1982) shows three hydrophobic sequence stretches (Fig. 3A, see arrows; Fig. 3B: 1, residues 32-52; 3, residues 346-368; 4, residues 373-390). The domain prediction program SMART predicts an additional membrane span comprising residues 309-331 (Fig. 3A,B, question mark). The sequences of all four putative transmembrane domains (TMDs) are predicted to form α-helices of sufficient length to span the membrane (Fig. 3C and supplementary material Fig. S3: TMD 1, 21 residues; TMD 2, 23 residues; TMD 3, 23 residues; TMD 4, 18 residues).

Most of the LUMA sequence comprises a hydrophilic domain between predicted TMD 1 and TMD 2 (Fig. 3A,B, residues 53-308). Within this domain, a large sequence segment (residues 116-164) is predicted by FoldIndex to be natively unfolded (Fig. 3D, residues 116-164). Since LUMA has no predicted signal peptide, this hydrophilic domain is most likely to reside in the ER lumen. The predicted topology of LUMA is summarized in Fig. 4A. To test this prediction, we first generated a series of LUMA constructs: a full-length LUMA with a C-terminal V5-tag and several truncation mutants carrying a C-terminal Myc-tag (Fig. 4B). In LUMAgal, the hydrophilic domain has been replaced by a part of β-galactosidase sequence, thereby removing epitope(s) recognized by anti-LUMA antibody. All the N-terminal truncation mutants were cloned in-frame behind the human growth factor signal peptide sequence to ensure correct insertion through the ER membrane (Fig. 4B). We then transcribed, and translated full-length and mutant 1-309 LUMA DNA in vitro in the presence of 35S-methionine and microsomal membranes, subjected the translated and membrane-inserted LUMA to proteolytic digestion by proteinase K, and analyzed the remaining fragments by SDS-PAGE and autoradiography. In our assay, a hydrophilic domain residing in the ER lumen would be protected from the proteolytic digestion by the microsomal membranes. Indeed, protease digestion causes the 52.5 kDa LUMA band (Fig. 4C, lane 1, a) to shift by 10 kDa (Fig. 4C, lane 2, b). Thus a 42.5-kDa fragment of LUMA is protected from digestion by membrane insertion. The simultaneous proteinase K digestion and membrane solubilization with Triton X-100 leads to almost complete loss of LUMA signal (Fig. 4C, lane 3). When using the truncation mutant consisting of residues 1-309 (Fig. 4C, lanes 4-6), the same experiment shows protection of a 38 kDa fragment from proteolytic digest: the 41.6 kDa LUMA band (Fig. 4C, lane 4, band c) shifts by 3.6 kDa (Fig. 4C, lane 5, band c). Bands e and f in lanes 5 and 6 originate from LUMA that has not been incorporated into microsomal membranes, whereas band d in lane 4 is probably a by-product of the translation (data not shown). Thus, we confirm that the hydrophilic domain of LUMA is localized to the ER lumen and prove that the first hydrophobic stretch in LUMA (Fig. 3B, residues 31-51) is indeed a TMD.
Since LUMA has up to three TMDs following the hydrophilic domain (Fig. 3B and Fig. 4B), its C-terminus may be directed either towards the ER-lumen or the nucleoplasm. To find out, we examined the accessibility of different antibody epitopes on transiently expressed, C-terminally-tagged LUMA variants (Fig. 5A-C) in digitonin-permeabilized cells. In contrast to Triton X-100, digitonin permeabilizes the plasma membrane but leaves intracellular membranes intact. We monitored permeabilization by staining for the cytoplasmic marker protein tubulin which, in contrast to the nucleoplasmic marker lamin B2, is accessible for antibodies in digitonin-permeabilized cells (Fig. 5A).

First, we used the anti-LUMA antibody raised against the hydrophilic domain to probe endogenous LUMA in HeLa cells. As expected, the anti-LUMA antibody requires permeabilization of cells with Triton X-100 to access its epitope(s) (Fig. 5A). To check the orientation of LUMA C-terminus, we repeated the permeabilization experiment on HeLa cells transiently expressing full-length LUMA and the LUMAgal mutant (Fig. 5B). LUMAgal is correctly targeted to the ER/INM, as the anti-Myc antibody stained both ER and the nuclear rim (Fig. 5B). In analogy to the previous experiment (Fig. 5A), the cells required permeabilization with Triton X-100 for anti-LUMA antibody to bind the endogenous LUMA as well as the expressed LUMA 1-400 (Fig. 5B). By contrast, the C-terminal tags of full-length LUMA and LUMAgal mutant are fully accessible already in digitonin-permeabilized cells (Fig. 5B): immunofluorescence staining with anti-V5 and anti-Myc antibodies generates a pattern that is in accordance with staining of the ER plus outer nuclear membrane. We conclude that the LUMA C-terminus is exposed to the cytoplasmic face of the ER and to the nucleoplasm when LUMA is enriched at the INM.

To validate our results and to investigate whether the second hydrophobic stretch is a true TMD, we repeated the permeabilization experiment on HeLa cells expressing N-terminally truncated LUMA (Fig. 5C). Both LUMA SP 53-309 and SP 53-200 [containing an N-terminal signal peptide (SP)] were correctly localized to the ER-lumen – their C-terminal Myc tag could be first detected after permeabilization with Triton X-100 (Fig. 5C). Also, emerin could first be detected after Triton X-100 permeabilization (we used the antibody FL254 raised against full-length emerin, which recognizes yet uncharacterized binding epitopes). By contrast, the C-terminus of the mutant SP 53-345, containing the second hydrophobic stretch of LUMA was already accessible in digitonin-permeabilized cells (Fig. 5C), indicating a cytoplasmic or nucleoplasmic orientation. Thus, the second hydrophobic stretch of LUMA spans the membrane. Since the extreme C-terminus of LUMA has the same orientation as its second TMD, we conclude that – as predicted – LUMA has four TMDs.

LUMA oligomerizes
While studying LUMA topology, we observed that endogenous LUMA was partially displaced from the INM in cells overexpressing the LUMAgal mutant (Fig. 5B). Thus, overexpression of the mutant had a dominant-negative effect on localization of wild-type LUMA. We hypothesized that wild-type LUMA and LUMAgal interact with each other and overexpression of the mutant forces wild-type LUMA from the protein network that normally keeps it at the INM. To test this, we expressed LUMA truncation mutants (Fig. 4B) in HeLa cells together with the V5-tagged full-length LUMA, and used cell lysates for co-immunoprecipitation experiments with anti-V5 antibody (Fig. 6A). Strikingly, endogenous LUMA (LUMAe) immunoprecipitated together with
LUMA at the inner nuclear membrane

the overexpressed V5-tagged LUMA (LUMA 1-400) in each sample (Fig. 6A, lanes 5-8). Additionally, both LUMAgal and LUMA 1-309 precipitated with full-length LUMA (Fig. 6A, lanes 10 and 8), indicating an interaction. By contrast, the deletion mutant consisting of the hydrophilic domain only (LUMA 53-309) did not interact with full-length LUMA (Fig. 6A, lane 7). Apparently, LUMA oligomerizes through its first TMD and/or the N-terminal nucleoplasmic tail (amino acid residues 1-31 are present in all three constructs containing TMDs).

For verification, we produced \( ^{35} \text{S}-\)labeled LUMA wild-type and truncation mutants, either alone or as a mix, by coupled in vitro transcription/translation in the presence of microsomal membranes. We then separated the potential protein complexes on 2D-BlueNative–SDS-PAGE and detected the \( ^{35} \text{S}-\)labeled proteins by autoradiography. Without treatment with SDS, proteins that are part of a complex are expected to co-migrate at a high molecular mass in the first dimension (Fig. 6B, hmw). Treatment with SDS should disrupt the high-molecular-mass complexes and reduce them to monomers. LUMA 1-400 (Fig. 6B, \( \text{<} \)) and LUMAgal (Fig. 6B, \( \cdot \)), both containing all TMDs, indeed formed such high-molecular-mass complexes in the absence of SDS. By contrast, the hydrophilic domain alone (LUMA 53-309, Fig. 6B, \( \circ \)) did not form any high-molecular-mass complexes, neither with LUMA 1-400 nor with itself, validating our immunoprecipitation data.

Transmembrane sequences target LUMA to the INM

To examine which parts of LUMA are responsible for its targeting to the INM, we transiently expressed LUMA 1-400, LUMAgal and the truncation mutants LUMA 1-200, LUMA 1-309 (Fig. 7B), SP 53-345, SP 53-200 and SP 53-309 (Fig. 5C) in HeLa cells, and examined their localization by immunofluorescence staining of the C-terminal Myc-tag. All LUMA fragments containing at least one TMD were targeted to the NE (Fig. 7, Fig. 5C). The fragments SP 53-200 and SP 53-309 were correctly inserted into the ER-lumen, but did not accumulate at the nuclear rim (Fig. 5C). The nuclear staining for fragment SP 53-309 (Fig. 5C) does not resemble the DAPI stain (not shown) and, thus, most probably reflects the concentration of ER-membrane layers in this particular plane of focus.

ER proteins are targeted to the INM by the virtue of their interactions with nuclear components (Ellenberg et al., 1997; Ohba et al., 2004; Lusk et al., 2007). In our particular experiment, NE-targeting of the LUMA fragments probably originates from a combination of interactions with an unknown ‘anchoring factor’ and oligomerization with endogenous LUMA. Endogenous LUMA would of course need the ‘anchoring factor’ in order to accumulate at the INM.

The N-terminus including the first TMD or the second TMD plus 14 nucleoplasmic amino acid residues are sufficient for INM targeting (Fig. 7B, Fig. 5C). The only feature these sequences have in common is the presence of the TMD-interaction motifs (Russ and Engelman, 2000): GxxxG in the first TMD and AxxxA in TMD 2. The AxxxA motif is also present in TMD 3 and TMD 4. Thus we assume that LUMA interacts with its ‘anchoring factor’ through transmembrane domains. Compared with LUMA 1-309, the shorter fragments LUMA 1-200 (Fig. 7B) and SP 53-345 (Fig. 5C) have a
more pronounced ER-localization. Similarly, LUMAgal has more pronounced ER-localization than full-length LUMA (Fig. 7B). Thus, the hydrophilic domain may contribute to a correct LUMA targeting when one or more TMDs are missing.

LUMA is involved in the structural organization of nuclear membranes

While investigating LUMA targeting, we were using emerin as a marker of INM (Fig. 7, Fig. 5C). Emerin is an interesting protein, because of its disease-relevance: emerin is displaced from the NE under certain pathological conditions (Bengtsson and Wilson, 2004). We noticed that in cells overexpressing any LUMA variant, distribution of emerin was drastically changed (Fig. 7, and supplementary material Fig. S4). This redistribution phenotype was highly variable, combining to different degrees mislocalization and accumulation of emerin in foci at or near the INM. The truncation mutant SP 1-200 appears most disruptive: it causes nuclear invaginations and bizarre nuclear shapes. Similar, but less extensive nuclear shape changes are caused by overexpression of LUMAgal (Fig. 5B and Fig. 7B). By contrast, LUMA 1-309, which has an intact N-terminus and the complete hydrophilic domain, has the least disruptive effect. The truncation mutant SP 1-200 appears most disruptive: it causes nuclear invaginations and bizarre nuclear shapes. Similar, but less extensive nuclear shape changes are caused by overexpression of LUMAgal (Fig. 5B and Fig. 7B). By contrast, LUMA 1-309, which has an intact N-terminus and the complete hydrophilic domain, has the least disruptive effect. The difference in nuclear shape phenotypes after overexpression of LUMA 1-200, LUMAgal and LUMA 1-309 implies that the highly conserved domain of unknown function DUF1625 (Fig. 3B) is important for maintenance of nuclear structure and/or shape. LUMAgal still has the C-terminal part of DUF1625 (TMD 2 and TMD3), which might explain its intermediate phenotype (between those of LUMA 1-200 and LUMA 1-309).

The redistribution and/or mislocalization of emerin could be a consequence of generally altered nuclear structure, or a specific consequence of disturbed LUMA-emerin-complexes. We think co-aggregation due to LUMA overexpression as the cause of mislocalization is not very likely, because not all LUMA foci contained emerin and vice versa. To investigate effects of LUMA on nuclear shape and emerin distribution under more physiological conditions, we downregulated LUMA in HeLa (Fig. 8) and NIH 3T3 cells (not shown) by using specific micro RNAs (miRNAs). The miRNAs were co-expressed with GFP, from the same vector. As negative control, we used a miRNA that is predicted not to block translation of any specific gene. After 3 days, the amount of LUMA was significantly reduced in more than 90% of GFP-expressing cells that co-express the LUMA specific miRNA Hmi48 (Fig. 8). Likewise, a mix of three different specific miRNAs for murine LUMA mRNA works in NIH 3T3 fibroblasts (data not shown). In LUMA downregulated cells, emerin was either significantly reduced, or mislocalized to different degrees with or without formation of foci (Fig. 8). Expression of lamin A/C (Fig. 8) and lamin B2 (not shown) was not affected. We conclude that, (1) downregulation of LUMA closely resembles the LUMA overexpression phenotype, implying that overexpression of LUMA disrupts endogenous LUMA functions and, (2) that, LUMA is important for keeping emerin at the INM.

LUMA interacts with emerin

To distinguish between an interaction with LUMA as a reason for emerin mislocalization and an indirect effect due to the gross changes of the NE architecture, we checked whether LUMA interacts with emerin or other INM-associated proteins in vivo (Fig. 9A). We immunoprecipitated either LUMA, emerin, lamin A/C, lamin B2 or MAN1 from undifferentiated C2C12 cells and searched
LUMA at the inner nuclear membrane

We found endogenous LUMA co-immunoprecipitating with full-length LUMA, but not with LUMAgal or LUMA 53-309. We conclude that only full-length LUMA is capable of efficiently forming LUMA-eminin complexes.

Discussion

LUMA is a unique protein integral to the nuclear membrane

Conservation of LUMA during evolution is striking. Whereas most characterized INM proteins appear first in metazoans and some do not appear until in vertebrates (LAP2 family) (Mans et al., 2004; Tzur et al., 2006), LUMA orthologs are found in all sequenced vertebrate and insect genomes, unicellular eukaryotes, some plants and even several bacteria. The amino-acid sequence of LUMA is remarkably conserved, even in bacteria. This allowed the definition of the PFAM domain DUF1625, which is only found in LUMA orthologs. It is not clear whether some bacteria acquired or conserved LUMA; however, the fact that they did hints towards a fundamental cellular function for LUMA.

A rather large part of the hydrophilic domain and of DUF1625 (residues 116-164) is predicted to be natively unfolded. Such sequences have a propensity to adopt random-coil conformation until either a ligand binds or a post-translational modification occurs, which in both cases may induce the formation of a defined tertiary structure (Fink, 2005). Thus, it is possible, that the highly conserved DUF1625 represents a binding site for yet unknown interaction partner(s) of LUMA. The DUF1625-binding partner may be the factor crucial for the maintenance of nuclear structure, as overexpression of mutants lacking the DUF1625 domain caused striking nuclear shape malformations.

Our extraction and localization experiments show that a fraction of LUMA is retained at the nuclear envelope. This LUMA pool displays extraction properties typical for an integral membrane protein of the INM. For example, LUMA enriches during preparation of nuclei and nuclear envelopes, just like the integral INM proteins LBR or LAP2* (Bailer et al., 1991; Foisner and Gerace, 1993), solubilization of LUMA immobilized at the NE requires both high ionic strength and detergent.

Interestingly, a considerable fraction of cellular LUMA is not anchored at the NE. This non-INM LUMA is easily solubilized by detergents even at low ionic strength and is most likely to be identical with the LUMA fraction seen in the immunofluorescence images as ER-staining. Partial ER-localization is not uncommon for INM proteins (Ohba et al., 2004; Östlund et al., 2006); in the case of LUMA, however, the non-INM immobilized fraction seems unusually large. Future studies will reveal whether keeping excess of LUMA in the ER enables the cell to keep a steady supply of LUMA for the INM, or whether LUMA has specific functions in the non-nuclear ER domains.

LUMA topology

According to TMD prediction algorithms, LUMA contains four TMDs. We could confirm the first and second predicted TMD as true membrane-spanning domains, using protease-protection and digitonin permeabilization assays. Furthermore, we could show that the N- and C-termini of LUMA both reside on the nucloplasmic face of the nuclear membranes. Predicted TMD 3 and TMD 4 most probably span the membrane as well, because both sequences have enriched by immunoprecipitation. Endogenous emerin co-immunoprecipitated with full-length LUMA, but not with LUMAgal or LUMA 53-309. We conclude that only full-length LUMA is capable of efficiently forming LUMA-eminin complexes.
a high hydropathy index and show a high similarity to transmembrane domains in ABC-transporters. Thus, our results implicate that LUMA spans the membrane four times.

The hydrophilic domain of LUMA resides in the lumen of the ER, which makes LUMA the first characterized INM protein that has most of its mass inside the ER lumen. This topology is in agreement with our prediction, because LUMA lacks an N-terminal signal peptide for ER export. Only little is known about the function of the lumenal parts of INM proteins. The SUN domains of Sun1 and Sun2, for example, reside in the ER lumen and mediate protein-protein interactions that connect the nucleus with the cytoskeleton (Crisp et al., 2006). Gp210, a pore membrane protein with the bulk of its mass in the ER lumen, anchors the NPCs in the NE. Antibody binding to Gp210 luminal domain in living cells interferes with nuclear pore dynamics and disrupts nucleocytoplasmic transport (Greber and Gerace, 1992; Drummond and Wilson, 2002). Finally, the luminal part of LAP1 is a substrate for the triple-A protein torsinA, mutations in which cause DYT1 dystonia (Goodchild and Dauer, 2004; Goodchild and Dauer, 2005). We looked for, but did not find any, evidence for an interaction between Sun1, Sun2 and LUMA. Also, LUMA is not a torsinA substrate (Goodchild and Dauer, 2005). Thus the specific functions for the luminal part of LUMA are yet to be discovered.

Fig. 8. Downregulation of LUMA. HeLa cells were transfected with either a control vector expressing a control miRNA or the vector construct Hmi 48 expressing a miRNA designed to knockdown human LUMA mRNA. 72 hours after transfection, cells were fixed and subjected to immunofluorescence staining of LUMA, emerin and lamin A/C. Transfected cells are easily detected by GFP coexpression. Scale bars, 5 μm.

Fig. 9. LUMA interacts with emerin. (A) Immunoprecipitation of different endogenous proteins from undifferentiated C2C12 cells with specific antibodies as indicated. Stars indicate antibody cross-reactive proteins, arrowhead points at IgGs covering presumably LUMA signal. Input of emerin results from a longer exposure. (B) Full-length LUMA 1-400, LUMA 53-309 or LUMAgal were overexpressed in HeLa cells and immunoprecipitated with antibodies against their C-terminal tags (V5 or Myc). Precipitates were analyzed for the presence of LUMA and emerin. LUMAe, endogenous LUMA; ●, LUMA degradation product (lanes 1 and 3, masked in lane 2 by LUMA 53-309); ★, IgG heavy chains; ◊, LUMA 53-309 in lane 5 and C-terminal fragment of LUMAgal in lane 6. (C) Model for LUMA-emerin complexes at the INM.
TMDs contribute to LUMA oligomerization
Interestingly, all transmembrane spans of LUMA contain either GxxG (TMD 1) or AxxA (TMD 2 to TMD 4) motifs, which are known to mediate oligomerization of membrane spanning α-helices (Russ and Engelmann, 2000). Our experimental data show that the TMDs, together with the short neutralplasmic sequences, are important for LUMA–LUMA interactions. Whereas Sun-domain proteins oligomerize through their SUN-domains (Crisp et al., 2006), nothing is known about oligomerization of other INM proteins. LUMA may thus be the first INM protein known to form homooligomers through its transmembrane domains.

Such LUMA oligomers may form a platform for formation or organization of protein complexes in the INM. A hint comes from testing the proteolytic resistance of LUMA in vitro and in vivo. Whereas in-vitro-translated LUMA and the purified LUMA hydrophilic domain show no resistance against proteases such as trypsin, pronase or proteinase K, a remarkably high amount of endogenous LUMA remains stable in the presence of proteinase K in Triton-X-100-solubilized cells (data not shown). Thus, either some post-translational modifications or LUMA interaction partners protect LUMA from proteinase K digestion. Since only a few residues of emerin reside in the ER lumen and in the membrane (27 out of 245 residues), it seems unlikely that emerin alone could provide such protection. Lamins have to interact with LUMA on the nucleoplasmic side and, therefore, also do not offer any protection for the bulk of LUMA. Thus, oligomeric LUMA at the NE is most likely to form stable protein complexes that involve not only emerin or lamins (Fig. 9C) but also other interaction partners yet awaiting identification.

LUMA targeting to the nuclear envelope
One TMD together with the hydrophilic domain are sufficient to target LUMA to the NE, although parts of the hydrophilic domain might contribute to or modulate LUMA targeting. Although LUMA targeting to the INM depends on A-type lamins, it could also be mediated by binding of LUMA oligomers to other INM proteins. Our experiments have shown that the N-terminus including the first TMD is already sufficient for oligomerization. The formation of mixed oligomers when overexpressing any TMD-containing LUMA-truncation mutant could explain the dramatic effects on the NE structure: the incorporation of truncated forms into LUMA oligomers would negatively interfere with LUMA functions, presumably by disrupting the interaction of LUMA with other proteins. In fact, the LUMA overexpression phenotype – at least for emerin – closely resembles that of LUMA downregulation. Overexpressing full-length LUMA, however, might sequester essential components of the NE into non-nuclear ER membranes and, thereby, affect NE structure in different ways, which would explain the observed variable phenotypes. Since overexpression of LUMA mutants has such a dramatic effect on NE structure, we propose that LUMA is essential for the organization of protein complexes in the nuclear membrane.

LUMA interacts with emerin
Overexpression of any LUMA constructs has dominant effects on emerin localization. The observed effects could be a consequence of disturbed binding between LUMA and emerin. We do not know whether the interaction between LUMA and emerin is direct, because we can only produce and purify the hydrophilic domain of LUMA as a soluble protein from bacteria. However, we do know that emerin binding to LUMA is specific: neither MAN1 nor LAP2β can bind LUMA (data not shown), although MAN1, Lap2β and emerin share many of their interactions partners (Zastrow et al., 2004; Bengtsson, 2007).

LUMA and tetraspanins may have similar functions
Tetraspanins are abundantly expressed integral membrane proteins that span the membrane four times and share several conserved residues. Many of these proteins are localized in the plasma membrane. They have large extracellular loops that mediate interactions with other membrane proteins, e.g. integrins, and contribute to oligomerization. The main oligomerization motifs of tetraspanins are the highly conserved TMDs. Mutations in TMDs disrupt the tetraspanin network and lead to dystrophic diseases (Stipp et al., 2003; Hemler, 2005).

In some aspects, LUMA shows remarkable similarity to tetraspanins, although – when judged by its primary structure – it does not belong to this protein family. Both LUMA and tetraspanins have a large extracellular or luminal domain contributing to binding other integral membrane proteins, both oligomerize through TMDs and both have the potential to disrupt membrane organization with catastrophic disorganization of the affected cell.

Two tissue-specific dystrophies map to the region on chromosome 3 where LUMA is located (3p25.1): the limb girdle muscular dystrophy LGMD1C (MIM number 601253) and dilated cardiomyopathy CMD1E (MIM number 601154). Variants of LGMD and CMD have been attributed to disturbed protein interactions at the NE (Rankin and Ellard, 2006). The LGMD1C has been linked to mutations in caveolin (Minetti et al., 1998); however, other proteins might also be involved. The cause of CMD1E is currently unknown. Thus, mutations in LUMA may, just as in tetraspanins, cause or contribute to dystrophic diseases restricted to a specific tissue.

Has LUMA a role in laminopathies?
Our results indicate that LUMA has an important role in maintaining NE structure by organizing protein complexes at the INM. Overexpression of full-length LUMA or one of several truncation mutants affect the distribution of emerin and might have some effect on chromatin distribution as determined by changes in the DAPI stained distribution of DNA in cells. Since the absence of emerin from the nuclear membrane due to degradation or mislocalization causes Emery-Dreifuss muscle dystrophy (EDMD) (Muchir et al., 2006), LUMA dysfunction could well be involved in the molecular mechanism leading to muscular dystrophy.

Although EDMD patients have emerin deficits in all cells, only the heart, tendons and skeletal muscles are pathologically affected. The exact molecular mechanism of EDMD is unknown, but it has been postulated that the lack of emerin at the nuclear rim alters the expression of genes relevant to the affected tissues (Bengtsson and Wilson, 2004). This hypothesis implies that emerin forms a variety of complexes at the nuclear periphery, a finding that, indeed, was described recently (Holaska and Wilson, 2007). It is possible that, in most cells, these protein complexes can carry out their functions even in the absence of emerin but that they cease to function if additional components are missing. Interestingly, the remarkably highly conserved protein LUMA, which is important for the correct localization of emerin, is not expressed in the skeletal muscle and only at a very low level in heart. It would be interesting to examine, whether reintroduction of LUMA in these tissues helps to alleviate the disease phenotype caused by mutant emerin in EDMD patients. Future studies will hopefully elucidate the role of LUMA in the pathological mechanisms leading to EDMD.
Materials and Methods

Bioinformatics

The hydropathy plot (Kyte and Doolittle, 1982) was obtained using OMMGA 2.0 (Oxford, Oxford, UK). Orthologs were identified using BLASTP or TBLASTN (http://www.ncbi.nlm.nih.gov/blast/). Genomic information was obtained via Entrez. Gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genbank) and UnGenie (http://www.ncbi.nlm.nih.gov/UnGenie/). The domain structure was predicted using SMART (Schulz et al., 2000), alignments were obtained using ClustalW and ESPpr, secondary structure was predicted with GOR I, HNN, SOPM and SOPMA (http://www.expasy.org). Natively unfolded LUMA sequences were predicted with FoldIndex at http://bpj.weizmann.ac.il/FoldIndex (Pritulsky et al., 2005).

Cell culture

N2a, CC12, NIH 3T3 and MEF cells were cultured in DMEM plus 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 μg/ml penicillin. HeLa cells in RPMI-1640 plus 10% FBS, 100 μg/ml streptomycin, 100 μg/ml penicillin, 2 mM L-glutamine and non-essential amino acids (Invitrogen, Karlsruhe, Germany), in a humidified atmosphere (5% CO2 at 37°C). LMNA−/− and wild-type MEF cells were a kind gift from Brian Burke, University of Florida.

Transfection

Cells were transfected at 50% confluency using poly-ethyleneimine (PEI, Sigma). For transfection of cells growing in a 3.4-cm plate, 5 μl PEI in 200 μl DMEM were incubated 30 minutes at room temperature, diluted with 1.8 ml DMEM, and used to replace the growth medium. After 5 hours, the transfection mix was replaced by growth medium. After another 20 hours, the cells were subjected to either cell extraction or immunofluorescence staining.

Cell extraction

Cells were washed once with ice-cold PBS and then extracted (1 hour, 4°C, gently agitation) by adding 1 ml Triton X-100 lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (w/v) Triton X-100, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μM MG-132 (Calbiochem, Schwalbach/Ts., Germany), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)). After a 10-minute centrifugation (14,000 g, 4°C) the supernatants were analyzed further.

Preparation of nuclei and nuclear envelopes from N2a cells

Nuclei from N2a-cells were prepared as described previously (Dreger et al., 2001). Nuclear envelopes were prepared according to Otto et al. (Otto et al., 1992).

DNA constructs

Full-length LUMA (1-400) in pcDNA3.1/V5-His TOPO (Invitrogen, Karlsruhe, Germany) has been described before (Dreger et al., 2001). LUMA-truncation mutants were constructed by amplifying the sequence encoding the mutant by PCR from full-length LUMA and subcloning the fragments into pcDNA3.1(−)myc/His C (Invitrogen, San Diego, CA). For N-terminally truncated clones, the signal peptide sequence of LUMA and subcloning the fragments into pcDNA3.1(−)myc/His C (Invitrogen, San Diego, CA). To construct the LUMAgal domain 3 [(residues 335-634, nucleotides 1003-1902 of the coding region (Jacobson et al., 1994)]. We amplified this region by PCR from the plasmid pHM830 (Sorg et al., 1999). All samples were then separated by BN–SDS-PAGE. Gel strips were transferred to nitrocellulose. The radioactively labeled LUMA and 100. After 30 minutes of proteolysis at 4°C, 2 μM PMSF and SDS-PAGE sample buffer, the other was prepared for BN–SDS-PAGE as described (Nicke et al., 1999). All samples were then separated by SDS-PAGE and transferred to nitrocellulose. The radioactively labeled LUMA and its fragments were visualized by autoradiography.

Interaction analysis by 2D-Blue Native (BN)–SDS-PAGE

Full-length and truncated LUMA was in-vitro-translated as a 35S-methionine-labeled protein in the presence of canine microsomes using the TNT Quick system (Promega GmbH, Mannheim, Germany) according to the manufacturer’s instructions. The resulting sample was divided into three aliquots: one was left untreated, one aliquot received 0.1 mg/ml protease K and one 0.1 mg/ml protease K and 1% Triton X-100. After 30 minutes of proteolysis at 4°C, 2 mM PMSF and SDS-PAGE sample buffer were added. The samples were heated for 5 minutes at 95°C, separated by SDS-PAGE and transferred to nitrocellulose. The radioactively labeled LUMA and its variants were generated, separately or in mixture and in the presence of canine microsomes, as 35S-methionine-labeled proteins as described above. The resulting samples were divided into two aliquots. One aliquot received the SDS-PAGE sample buffer, the other was prepared for BN–SDS-PAGE as described (Nicke et al., 1999). All samples were then separated by BN–SDS-PAGE as first dimension. Gel strips cut from the first-dimension gel were transferred to SDS–PAGE conditions and separated by SDS–PAGE as second dimension. After blotting onto PVDF membranes, the radioactively labeled proteins were visualized by autoradiography.

We are grateful to Inka Fedke, Hermann Bayer and Giampiero Bandini for their technical support. We thank Chris Weise and Jan Börgemann for critical reading of the manuscript. We thank Ferdinand Huchó, Petra Knaus, Gerd Multthaup, and Katherine L. Wilson for their generous support. This work was supported by the grant Hu 146/22-1 (H.O. and Ferdinand Huchó), by the post-doctoral-
fellowship grant of the DFG (L.B.) and by the Fonds der Chemischen Industrie (F.H.).

References


Supplemental figure S3. Secondary structure prediction for LUMA.
Individual predictions and consensus of the secondary structure prediction for LUMA obtained by GOR1, NNpredict, HNN, SOPM, and SOPMA. Grey: α-helix, black: β.
A

m.w. (kDa)
67 -
49 -
36 -
29 -
24 -
20 -
1 2 3 4 5

LUMA

1  Serum
2  Pur. Ab. 1:1,000
3  Pur. Ab. 1:2,000
4  Pur. Ab. 1:10,000
5  Control

B

LUMA  DAPI

Control

1:1000

1:500

1:100
<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Oligo</th>
<th>Reverse Oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>LUMA (1-200)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (1-309)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (53-200)</td>
<td>5'-ATTCCTCTAGAATGAACGAGGGCCGAGCACTG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (53-309)</td>
<td>5'-ATTCCTCTAGAATGAACGAGGGCCGAGCACTG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (53-345)</td>
<td>5'-ATTCCTCTAGAATGAACGAGGGCCGAGCACTG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (LUMA 1-165)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (LUMA 306-400)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>LUMA (β-D-galactosidase domain 3)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>Signal peptide</td>
<td>5'-CTAGCGCCACCACATGGCTACTTGCTCCCCAGAACTTCTTTTGCTGCTTTGCTTTC</td>
<td>5'-GGCTTTGGTGTTGTCCAAGCGAACTATTCTGCTCTTGCCTTGGCTTGTCTT</td>
</tr>
<tr>
<td></td>
<td>5'-CTAGCGCCACCACATGGCTACTTGCTCCCCAGAACTTCTTTTGCTGCTTTGCTTTC</td>
<td>5'-GGCTTTGGTGTTGTCCAAGCGAACTATTCTGCTCTTGCCTTGGCTTGTCTT</td>
</tr>
<tr>
<td></td>
<td>5'-CTAGCGCCACCACATGGCTACTTGCTCCCCAGAACTTCTTTTGCTGCTTTGCTTTC</td>
<td>5'-GGCTTTGGTGTTGTCCAAGCGAACTATTCTGCTCTTGCCTTGGCTTGTCTT</td>
</tr>
<tr>
<td></td>
<td>5'-CTAGCGCCACCACATGGCTACTTGCTCCCCAGAACTTCTTTTGCTGCTTTGCTTTC</td>
<td>5'-GGCTTTGGTGTTGTCCAAGCGAACTATTCTGCTCTTGCCTTGGCTTGTCTT</td>
</tr>
<tr>
<td></td>
<td>5'-CTAGCGCCACCACATGGCTACTTGCTCCCCAGAACTTCTTTTGCTGCTTTGCTTTC</td>
<td>5'-GGCTTTGGTGTTGTCCAAGCGAACTATTCTGCTCTTGCCTTGGCTTGTCTT</td>
</tr>
<tr>
<td>LUMA 53-309 (for antibody production)</td>
<td>5'-ATTCCTCTAGAATGGCAGCGAACTATTCCAG</td>
<td>5'-ATTCCAAGCTTTCAATCTTGTCAATTGAGCCTG</td>
</tr>
<tr>
<td>hLUMA 1-400</td>
<td>5'-ATGGCCGCGAATTATTATACCAGTACCAGTACC</td>
<td>5'-CCAGTCTCAACAGGTCAGATTCCCTG</td>
</tr>
<tr>
<td>Hmi48</td>
<td>5'-TGCTGATAAAGGTGTAGCCACATGAACGTTGTGGCCACACGTTGTCAATGCTCACCATAT</td>
<td>5'-CTGATAAAGGTGTAGCCACATGAACGTTGTGGCCACACGTTGTCAATGCTCACCATAT</td>
</tr>
</tbody>
</table>