universität wien

DISSERTATION

Characterization of Novel Members of the LEM-Domain Containing Protein Family in Mammalian Cells

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer.nat.)

Verfasser:	Mag. Andreas Brachner
Matrikel-Nummer:	a9702216
Dissertationsgebiet (lt. Studienblatt):	Molekulare Biologie
Betreuerin / Betreuer:	Univ.-Prof. Dr. Roland Foisner

Wien, am 28. Jänner 2008

Identification and Functional Characterization of Novel Members of the LEM-Domain Containing Protein Family

Cover Image "Journal of Cell Science" 118, Dec 2005: Overexpression of the novel inner nuclear membrane protein hLEM2 leads to the formation of tubular connections between nuclei of adjacent cells that probably originate from a defect in postmitotic nuclear membrane reassembly. The confocal image shows a triple labeling for LEM2 (green), actin (red) and DNA (blue). See article by Brachner et al. (pp. 5797-5810).

Danksagung

Ich möchte mich mit diesen Zeilen bei all jenen Menschen bedanken, die auf die eine oder andere Weise zum erfolgreichen Abschluß meiner Dissertation beigetragen haben.

Einen großen Anteil am Gelingen dieser Arbeit haben naturgemäß meine beiden Betreuer,
Prof. Roland Foisner und Dr. Josef Gotzmann,
die mich während der gesamten Entstehungszeit der Dissertation mit Rat und Tat (sowie Kaffee und Lebensweisheiten) unterstützt haben.

Weiters danke ich den Mitgliedern meines PhD-Komitees,
Prof. Christian Seiser und Prof. Pavel Kovarik
für die alljährliche, rege Diskussion meiner aktuellen Ergebnisse und Versuchspläne.

Des weiteren ein großes Danke an alle Kollegen in der Arbeitsgruppe Foisner und am Institut für Biochemie – durch die stets harmonische und positive Atmosphäre, hat mir die Arbeit im Labor (fast) immer großen Spaß gemacht.

Und nicht zuletzt haben auch meine Familie und meine Freunde aus anderen Lebensbereichen und insbesondere meine Liebste, Chantal, maßgeblich zur Fertigstellung meiner Doktorarbeit beigetragen.

Dafür ebenfalls ein herzliches Danke an dieser Stelle.

Table of Contents

1. Abstract / Kurzfassung 9

2. Introduction

2.1 The Nucleus – Evolutionary Aspects 11
2.2 The Nuclear Structure Embedded in Cellular Networks 13
2.3 The Nuclear Lamina 18
2.4 Human Diseases Associated with the Nuclear Envelope 20
2.5 The LEM Domain Containing Protein Family 22
2.6 Barrier-to-Autointegration Factor – a BAFfling Little Protein *(published, 2007)* 25
2.7 Aims of the Thesis 33

3. Results & Discussion

3.1 Evolution of the LEM Domain Containing Protein Family 35
3.2 LEM2 41
3.2.1 LEM2 is a Novel MAN1-Related Inner Nuclear Membrane Protein Associated with A-type Lamins *(published, 2005)* 42
3.2.2 Localization and Phenotypical Consequences of Expressed LEM2 Protein Domains *(unpublished)* 56
3.2.3 Yeast *SRC1* (*HeH1*) and Human LEM2 are Orthologous Proteins with Functional Conservation Throughout Evolution *(unpublished)* 71
3.2.4 LEM2, Conclusions & Outlook 79
3.3 LEM3 81
3.3.1 LEM3 (ANKRD41) is a Novel LEM-Domain Protein that Shuttles Between Nucleus and Cytoplasm and Affects BAF *(submitted)* 82
3.3.2 LEM3, a Novel Player in DNA Recombination/Damage Response? *(unpublished)* 111
3.3.3 LEM3, Conclusions & Outlook 122

4. References 123

5. Appendix

Abbreviations 139
Curriculum Vitae 141
Publications 142

1. Abstract

The nucleus in eukaryotic cells is enclosed by a nuclear envelope consisting of an inner and outer membrane, nuclear pore complexes, and in metazoans a filamentous lamina meshwork underlying the inner nuclear membrane. The lamina consists of intermediate filament-type proteins, the lamins, and numerous integral inner nuclear membrane proteins. Among these, a family of membrane proteins contain a conserved structural motif, called LEM domain (LAP2, Emerin, MAN1), which interacts with the DNA binding molecule BAF (barrier-to-autointegration factor). LEM proteins have been implicated in chromatin organization and gene expression control and have been linked to a heterogeneous group of inherited human diseases, collectively termed "envelopathies". This PhD thesis describes the identification and initial characterization of two novel LEM proteins, LEM2 and LEM3.

LEM2 is ubiquitously expressed in many tissues and cell types and is closely related to MAN1 in primary sequence and domain topology. Complementation assays revealed that LEM2 is functionally conserved from yeast to man. In mammalian cells, it localizes at the inner nuclear membrane, interacts directly with A-type lamins and with BAF, and requires lamin A/C for nuclear envelope localization. The lamin A/C interaction domain was mapped to the N-terminus, while the C-terminus contains a conserved DNA binding motif. The latter was required for the ability of overexpressed LEM2 fragments to form patches at the nuclear envelope that recruit lamin A and lamin A-binding proteins, but exclude lamin B and associated proteins. Our data suggest a role of LEM2 in the spatial organization of protein complexes at the nuclear envelope and in chromatin organization at the nuclear periphery.

LEM3 is primarily expressed in hematopoietic tissues such as bone marrow, thymus and spleen, and in lymphoma-derived cell lines, suggesting a B-cell related function. Analysis of LEM3 domain topology revealed a cluster of Ankyrin repeats at the N-terminus and a conserved C-terminal GIY-YIG motif previously described in proteins with nuclease activity. I identified two LEM3 splice-isoforms lacking parts of the LEM domain. Unlike these isoforms full length LEM3 bound BAF. LEM3 misses a transmembrane domain and was found to shuttle between nucleoplasm and cytoplasm. In human cells ectopic LEM3 co-localizes with cytoplasmic actin filaments, while it is in nuclear splicing speckles upon pharmacological inhibition of nuclear export. Ectopic expression of LEM3 in the nucleus causes a mislocalization of BAF, cell cycle arrest, and activation of the ATM-dependent DNA damage pathway. We propose that LEM3 may be involved in DNA recombination or repair pathways.

1. Kurzfassung

Der Zellkern, das charakteristische Merkmal eukaryotischer Zellen, wird von einer Kernhülle eingeschlossen, die aus einer doppelten Lipidmembran mit eingefügten Kernporenkomplexen und einer filamentösen Kernlamina in mehrzelligen Arten, die der inneren Kernmembran anliegt, besteht. Mehrere integrierte innere Kernmembranenproteine enthalten ein spezifisches Strukturmotiv, die sogenannte LEM-Domäne (LAP2, Emerin, MAN1), die das DNA-bindende Molekül BAF (barrier-to-autointegration factor) bindet. Mutationen in LEM Proteinen wurden mit humanen Pathologien assoziiert, die in der heterogenen Gruppe der sogenannten "Envelopathien" zusammengefaßt werden. Zwei neue LEM Proteine, LEM2 und LEM3, wurden in der vorliegenden Dissertation identifiziert und analysiert.

LEM2 ist ubiquitär in Geweben und Zelltypen exprimiert und ist sowohl bezüglich der Primärsequenz als auch der Domänen-Topologie eng mit MAN1 verwandt. Komplementations-assays zeigten, daß Funktionen von LEM2 von der Hefe bis zum Menschen konserviert sind. In Säugetierzellen lokalisiert LEM2 an der inneren Kernhülle, interagiert direkt mit Lamin A/C und BAF und benötigt A-typ Lamine für die Lokalisation an der Kernmembran. Die Lamin A/C-interagierende Region wurde auf einen Teil des N-Terminus eingegrenzt, wohingegen der C-Terminus ein DNA-Bindungsmotif enthält. Letzteres ist essentiell für überexprimierte LEM2-Fragmente um Komplexe an der Kernhülle zu formen, die Lamin A und Lamin A-assoziierte Proteine rekrutieren können, jedoch Lamin B und Lamin B-assoziierte Protein exkludieren. Unsere bisherigen Daten weisen auf eine Rolle von LEM2 in der räumlichen Organisation von Komplexen an der Kernhülle und in der Chromatinorganisation an der Kernperipherie hin.

LEM3 wurde vor allem in hämatopoietischen Geweben gefunden, so etwa im Knochenmark, Thymus und Milz, sowie in Lymphoma. Eine Analyse der LEM3 Domänenstruktur zeigte eine Gruppe von Ankyrin-Repeats am N-terminus des Proteins sowie ein evolutionär konserviertes GIY-YIG Motif innerhalb des C-Terminus welches zuvor in verschiedenen Proteinen mit Nukleasefunktion beschrieben wurde. Desweiteren habe ich zwei LEM3 Splice-Isoformen identifiziert bei welchen Teile der LEM Domäne fehlen, wobei diese im Gegensatz zum vollständigen LEM3 BAF nicht binden können. LEM3 enthält keine Transmembran-Regionen und wurde als Kern/Zytoplasma-„Shuttling"-Protein identifiziert. In menschlichen Zellen kolokalisiert LEM3 mit cytoplasmatischen Aktin-Filamenten, während es nach pharmakologischer Inhibierung des Kernexports in nukleären "Splicing-Speckles" zu finden ist. Ektopische Expression von LEM3 führte zu einer Mislokalisation von BAF, Zellzyklusarrest und Aktivierung des ATM-abhängigen DNA-Schädigungs-Signalweges. Wir postulieren eine Funktion von LEM3 in der DNA Rekombination oder im DNA Reparatur Signalweg.

2. Introduction

2.1 The Nucleus – Evolutionary Aspects

The nucleus, described first by the Scottish botanist Robert Brown in 1832, represents the largest subcellular compartment within eukaryotic cells and the presence of a nucleus is the major feature that distinguishes eukaryotes from the prokaryotic regna *Eubacteria* and *Archae*. Two hypotheses describe when and how the nucleus may have originated during evolution. According to the "endosymbiont hypothesis" (Fig. 1) the nucleus (as well as other cell organelles enclosed by a double membrane) originated by the incorporation of an early Archae bacterium into a eubacterium by an endocytosis process thereby forming a proto-eukaryotic cell (Lake and Rivera 1994; Lopez-Garcia and Moreira 2006). Furthermore, it was proposed that the outer nuclear membrane (ONM) was established with the formation of the endoplasmic reticulum (ER) by fusion of internal cellular membranes of the host cell (Lopez-Garcia and Moreira 2006).

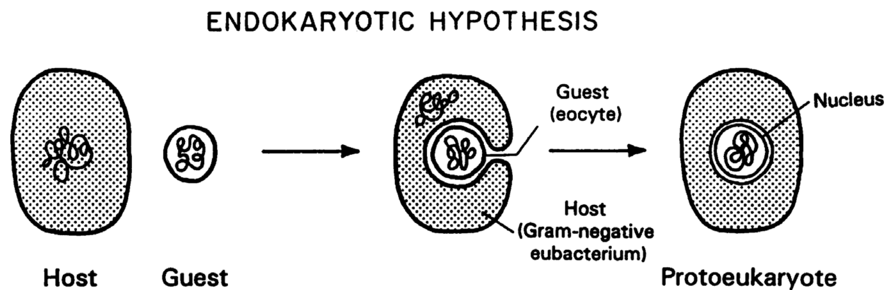


Figure 1: The evolutionary origin of the nucleus according to the endosymbiont hypothesis. Adapted from (Lake and Rivera 1994).

The endosymbiont model is also used to explain the origin of mitochondria and chloroplasts (Lang *et al.* 1999). Regarding the biochemical composition of the different organelle envelopes however, clear differences between the inner versus outer membrane sheets were noticed. Whereas the outer membranes of mitochondria and chloroplasts, like the endoplasmic reticulum, contain cholesterol, diphosphatidylglycerin (synonymous: cardiolipin) is the major phospholipid within their inner membranes is (Schlame *et al.* 2000), which is also a component of the today's bacterial cell membrane. In contrast, such a biochemical difference was never reported between the inner and outer nuclear membrane of eukaryotic nuclei. According to lipid and protein composition, the nuclear envelope is more closely related to the ER membranes than to the inner membranes of mitochondria (Guidotti 1972). Therefore the endosymbiotic origin of mitochondria and chloroplast is mostly accepted today, but a similar scenario for the evolution of the nucleus is under heavy debate. It is also unclear how nuclear pore complexes, huge, essential protein assemblies present in all cell nuclei from yeast to man could have evolved. Nevertheless,

it may be possible that endosymbiotic bacteria may have existed over a long time as a rather independent symbiont, and nuclear pore precursors were established. Consequently, one would have to assume that during this time, the genetic information of the host cell was concentrated in this protonucleus and metabolic activities were transferred to the cytoplasm, which is hard to explain.

The second hypothesis, which emerged some years ago, suggests a stepwise co-evolution of the nuclear envelope (NE) and the nuclear pore complex (NPC).

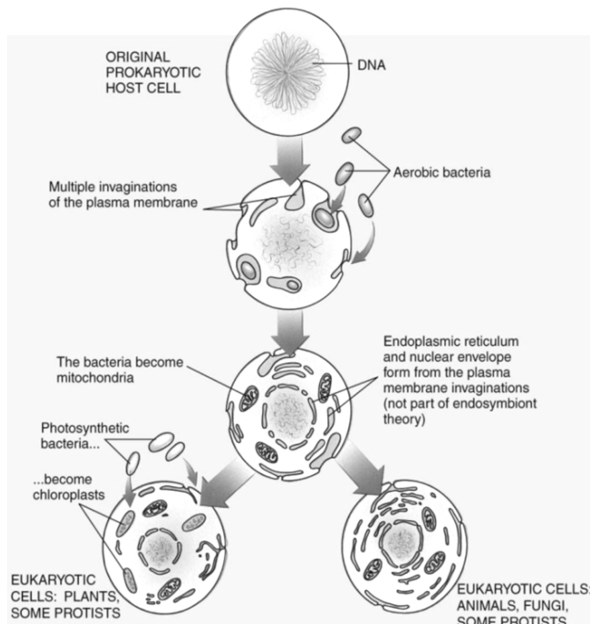


Figure 2: The origin of the nucleus according to the hypothesis of a stepwise fusion of inner cell membranes. Adapted from "Basic Concepts in Biology", Brooks-Thomson, 2003

From our current view of evolution, this scenario seems more conceivable than the endosymbiose model. The quintessence of this hypothesis is the gradual fusion of internal cellular membrane systems over a long time period, finally giving rise to the ER of eukaryotic cells, and additionally resulted in a completely enveloped genome (Fig. 2+3) (Martin and Koonin 2006).

The fact that in eukaryotic cells the ER is continuous with the outer nuclear membrane fits to the view of a nuclear envelope that derived from the same membranes, which gave rise to the ER. Additionally it was suggested that *planctomycetes*, an exceptional class of bacteria that contains in contrast to all other known bacterial classes internal membranes, partially enclosing the bacterial genome, may represent such early stages of nuclear evolution (Fuerst 2005; Martin and Koonin 2006; Pennisi 2004).

A pathbreaking study by Devos and colleagues postulates that the key developmental step for the evolution of all internal membrane structures and subsequently for the formation of the nuclear envelope and nuclear pore complex in todays eukaryotic cells was the generation of protein modules, which allowed bending and stabilizing sharp membrane curvatures (Fig. 3). In support of this model, some of the coating proteins in the yNup84/vNup107–160 subcomplex of the NPC are also found in coated vesicles and in both cases they are needed to stabilize the membrane curvature (Devos *et al.* 2004).

Independent of the origin of the nucleus, its formation led to the spatial separation of transcription and translation. The strict separation of transcription and translation machinery had fundamental implications for the regulation of gene expression and DNA replication.

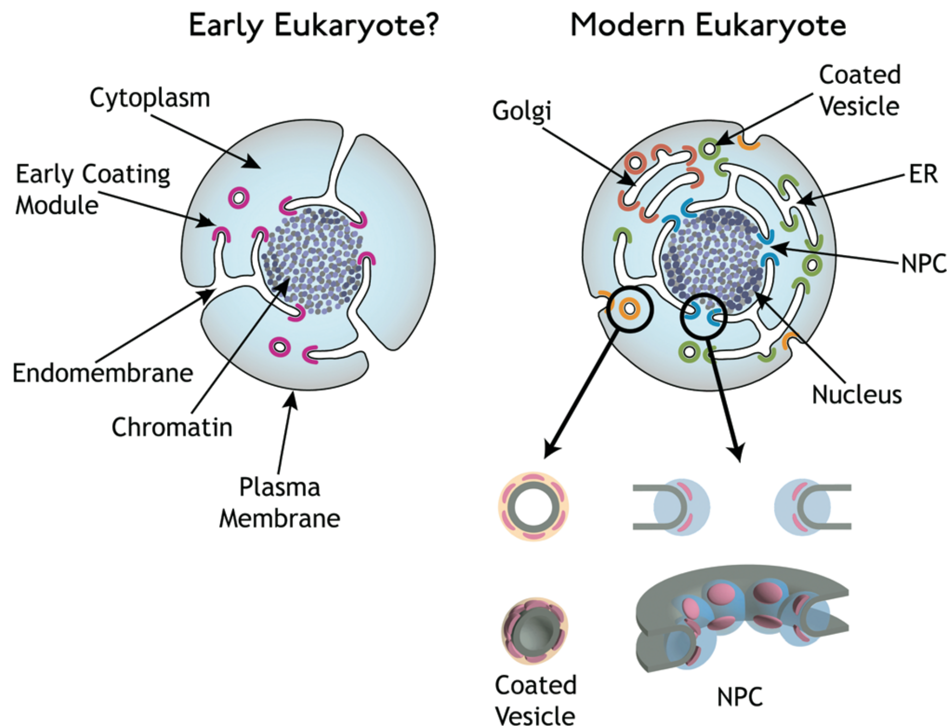


Figure 3: A probable model for the evolution of the nuclear pore complex and the key role of coating modules. Adapted from (Devos et al. 2004).

This compartmentalization of cellular functions was complemented by the formation of two major organelles, mitochondria and in plants additionally the chloroplasts through endosymbiosis (de Duve 2007). The uncoupling of transcription and translation and the location of distinct cellular functions to separate compartments is thought to be an essential prerequisite for the evolution of multicellular organisms.

2.2 The Nuclear Structure Embedded In Cellular Networks

The nucleus of all eukaryotic cells contains three major structural compartments. The nucleolus, mainly harboring ribosome biosynthesis (Hernandez-Verdun 2006; Raska *et al.* 2006), the nuclear envelope (NE), consisting of a lipid bilayer with inserted nuclear pore complexes (NPC), and the nucleoplasm (or nuclear interior) containing the chromatin and a multitude of subnuclear protein complexes with defined functions. Metazoan organisms contain additionally a filamentous structure at the NE, which underlies the inner nuclear membrane (INM), the so-called nuclear lamina. The nuclear lamina is thought to confer mechanical stability to the nucleus and provides an interaction platform for nuclear proteins and chromatin at the nuclear

periphery. Functional and structural data have been described in much detail for the nucleolus, the NE and the NPC, whereas the organization of the nuclear interior is still poorly understood.

The NE is composed of inner and outer membrane sheets, which are separated by the perinuclear space and are linked at the NPCs (Fig. 4). Inner and outer nuclear membrane (INM, ONM) differ significantly in their composition of resident proteins and fulfill very different functions. The ONM belongs morphologically – and likely also evolutionary (see also chapter 2.1) – to the rough ER (rER) with which it is continuous.

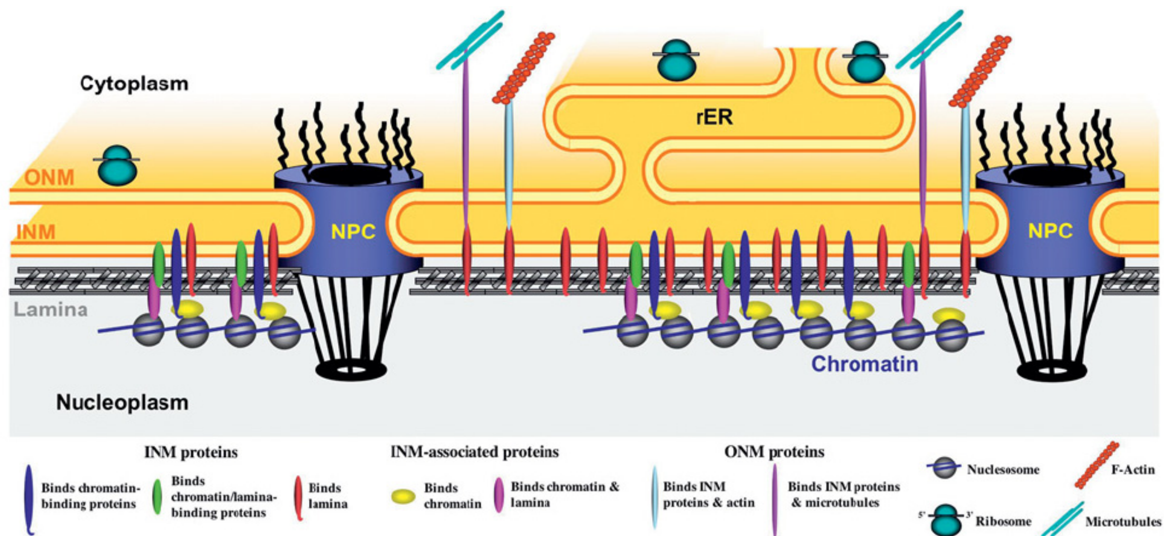


Figure 4: The nuclear envelope and its connection to the rough ER, the nuclear pore complex and the nuclear lamina. Adapted from (D'Angelo and Hetzer 2006).

Consequently, not only the membranes, but also the perinuclear and the inner space of the ER are forming a continuous compartment. Recently it was reported that TorsinA and TorsinB, both resident proteins of the ER lumen, are also found in the perinuclear space and may interact with proteins of the INM and ONM (Hewett *et al.* 2004). Despite its continuity with the ER, the ONM also contains specific proteins, including the family of Nesprin proteins, which establish a link between proteins of the INM and the cytoplasmic actin cytoskeleton and microtubules (Crisp *et al.* 2006; D'Angelo and Hetzer 2006; Hetzer *et al.* 2005; Vlcek *et al.* 2001; Wilhelmsen *et al.* 2006) (Fig. 5). This connection is established through specific interaction of two conserved protein domains in the perinuclear space: At the INM the C-terminal domain of SUN1 and SUN2 (the eponymous SUN domain (Sad1-UNC84 homology)), interacts with the N-terminal KASH (Klarsicht, ANC1, SYNE1 Homology) domain of Nesprin proteins, which reside in the ONM (Tzur *et al.* 2006). As a completely closed NE would prevent any traffic of molecules between nucleus and cytoplasm, NPCs', representing huge, symmetrically assembled protein complexes, form channels facilitating the molecular communication between these two cellular compartments. The NPC of higher eukaryotes consists of three super-imposed circular

structures: A cytoplasmic-, spoke- and nucleoplasmic ring, tightly fitting with the inner and outer nuclear membranes which are bent and fused at the spoke ring (Fig. 6).

In contrast to the metazoan NPC, the yeast NPC consists only of a single ring structure, which is homologous to the spoke ring. At the nucleoplasmic side of all NPCs, eight nuclear filaments are

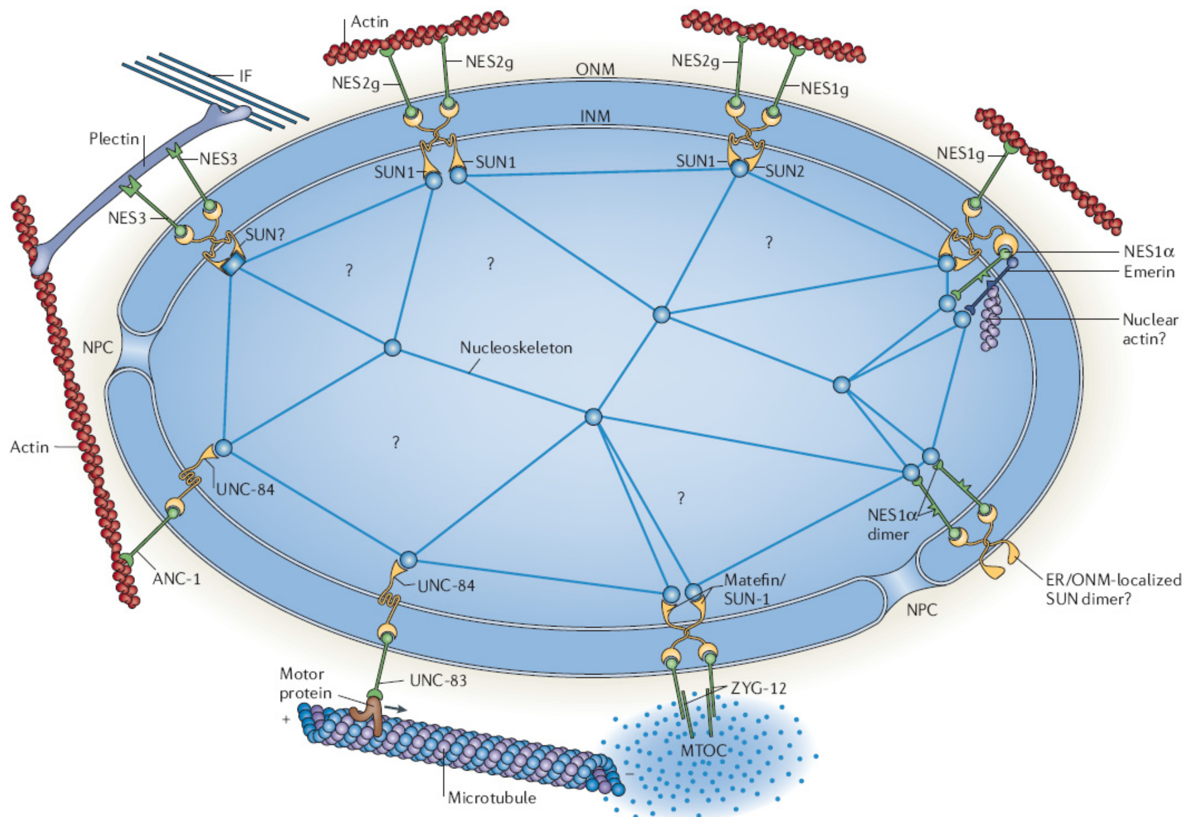


Figure 5: Nuclear envelope and cytoskeleton form an integrated network. Adapted from (Tzur et al. 2006).

protruding into the nuclear interior and are hold together by a distal protein ring, forming the so-called "nuclear basket". The cytoplasmic ring of the NPC is likewise decorated by eight filaments extending into the cytoplasm. Both, the nuclear basket as well as the cytoplasmic filaments have been reported to be involved in mRNA surveillance and export regulation (Forler *et al.* 2004; Lewis *et al.* 2007). Typically, a mammalian nucleus contains roughly 2000 NPCs, although the number may vary significantly depending on the physiological state of the cell (Adam 2001). NPCs permit passive diffusion of proteins smaller than 40kD but restrict passive movement of molecules with a higher molecular weight across the NE. The transport of these molecules depends on active transport mechanisms through the NPC. Both, the overall structure of the NPC and the molecules involved in active nuclear transport, are well conserved throughout the eukaryotic kingdoms and can be found already in unicellular organisms. Integral membrane proteins of the INM represent a special case in terms of nuclear import as these proteins are synthesized into the ER membrane and stay membrane-bound.

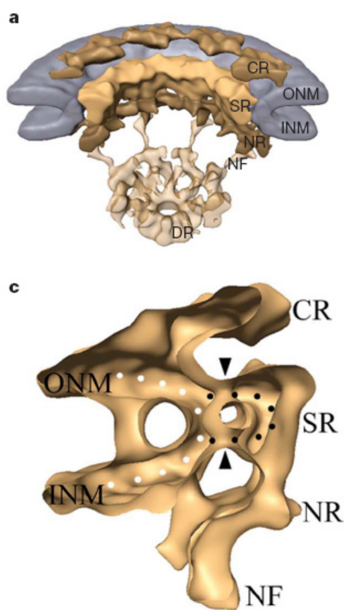


Figure 6: The NPC imaged by cryo-electron microscopy. Adapted from (Beck et al. 2007). Cytoplasmic- (CR) Spoke- (SR) and Nucleoplasmic Ring (NR), Nuclear Filaments (NF), Distal Ring (DR).

Several possibilities for the transport mechanism of integral membrane proteins to the INM have been suggested. A passive diffusion along the membrane continuities and subsequent retention by specific interactions was assumed previously. Recently, it was shown that integral proteins of the INM translocate along the membrane continuity at the NPC from the outer to the INM, but these proteins have to contain a nuclear localization signal (NLS) and use an active transport mechanism (King *et al.* 2006; Lusk *et al.* 2007). Integral INM proteins are retained at the nuclear periphery by specific interactions with chromatin, the nuclear lamina or other INM proteins. Whereas the main components of the NE and NPC as well as the nuclear lamina have been studied intensely, the structural and biochemical nature of the nuclear interior is still poorly defined. Persistently the existence of a "nuclear matrix" was proposed (Pederson 2000; Verheijen *et al.* 1988) mainly by use of transmission electron microscopy (Fig. 7) or in living cells (Fig. 8). However, the actual nature of this postulated protein matrix, which is supposed to be intermingled with chromosomes, various RNA species, hRNPs (heterogeneous RiboNucleoProteins), snRNP (small nuclear RiboNucleoProteins) and subnuclear bodies could not be determined on a biochemical level so far. It is still an open question whether this matrix is determined by the chromatin, by unknown nuclear matrix proteins or a combination of both (Nalepa and Harper 2004; Nickerson 2001; Pederson 2000). Independent of the existence of a nuclear matrix the nuclear interior seems to be heavily structured. Several studies presented evidence that individual chromosomes are not distributed randomly within the nucleus, but are assigned to specific areas, dependent on the cell type and physiological state of the cell. These defined regions occupied by specific chromosomes are called "chromosome territories" (Foster and Bridger 2005; Lanctot *et al.* 2007) and are thought to have fundamental effects on gene expression patterns. Extrinsic signals are thought to induce the translocation of specific chromosome territories to other intranuclear sites, facilitating the transcriptional activation or silencing of specific chromosomal regions (Bartova *et al.* 2002; Kuroda *et al.* 2004).

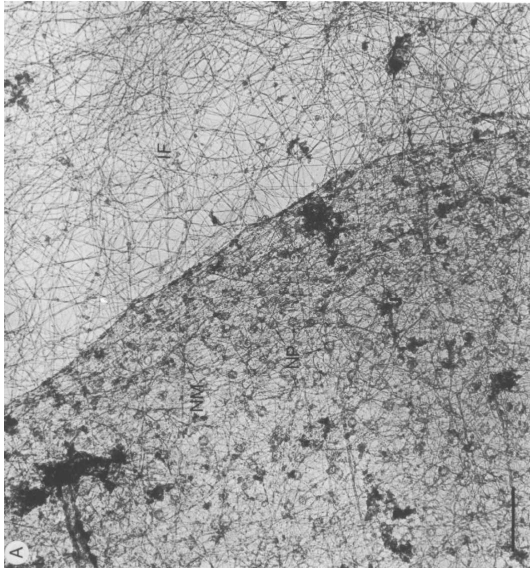


Figure 7: Electron micrograph of cytoplasmic filaments and the nuclear matrix of a human cell. Adapted from (Verheijen et al. 1988). Intermediate filaments (IF), nuclear matrix (NM), nuclear pore (NP).

Besides the chromosome-territories several types of "nuclear bodies" were identified over the past decades including Cajal bodies (Cioce and Lamond 2005), Nuclear Speckles (Lamond and Spector 2003) and PML bodies (Dellaire and Bazett-Jones 2004). Each of these discrete structures contain a specific set of proteins, chromatin and RNA molecules and fulfill particular functions in mRNA splicing and processing, DNA damage response and repair, micro-RNA processing and DNA replication (Lamond and Sleeman 2003; Lamond and Spector 2003; Matera 1999; Spector 2001). Most nuclear bodies are highly dynamic structures that are able to move within the nucleoplasm but assemble or disassemble according to the current requirements of the cell (Lamond and Sleeman 2003).

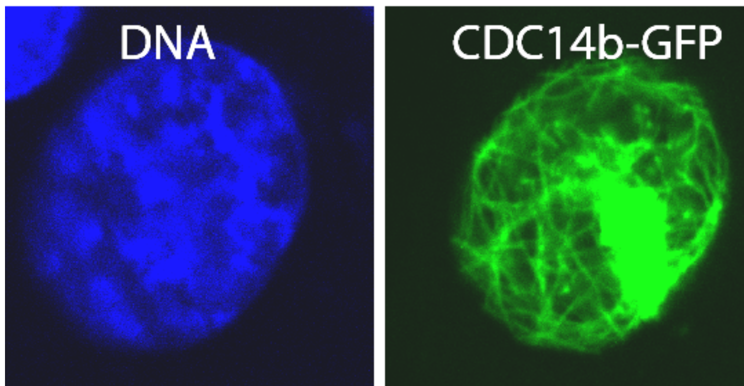


Figure 8: Evidence for a nuclear matrix in living cells, expressing GFP-tagged CDC14b (Nalepa and Harper 2004).

All together, the eukaryotic nucleus represents much more than a simple storage place for the genetic library of the cell. It is a highly organized and dynamic organelle with a plethora of subnuclear compartments regulating, organizing and integrating the different extrinsic and intrinsic signals in the "command centre" of the cell.

The big challenge in the future will be to draw a holistic concept of how the nucleus with all its different functions is organized at the molecular level and how the individual structural components organize and regulate this highly complex interplay of specific functions.

2.3 The Nuclear Lamina

The nuclear lamina, a filamentous protein network, was identified underneath the inner nuclear membrane of eukaryotic cells, with the exception of unicellular species (Aebi *et al.* 1986; Bridger *et al.* 2007; Foisner 2001; Gruenbaum *et al.* 2003) (Fig. 9).

There is no direct proof yet of a functionally related structure present in unicellular eukaryota like yeast or dinoflagellates, although previous studies claimed the existence of a lamina-like structure, mainly based on antibody cross-reactivity (Enoch *et al.* 1991; Galcheva-Gargova and Stateva 1988; Georgatos *et al.* 1989; Minguez *et al.* 1994). The nuclear lamins, which build the nuclear lamina in higher eukaryotes, are classified as type V intermediate filaments (Table 1) and represent the only filamentous protein structures within the nucleus known so far. The evolutionarily "earliest" organism with a clear, defined nuclear lamina is the nematode *Caenorhabditis elegans*. The nuclear lamina of *C.elegans* is composed of a single type of lamin, considered an equivalent to higher eukaryotes' B-type lamins, but more likely may be an ancestral lamin that split up in A-and B-type lamins later during evolution.

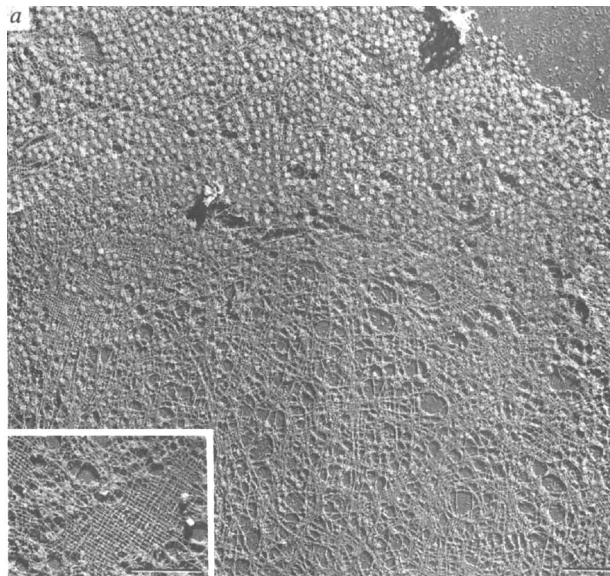


Figure 9: The Nuclear Lamina of a Xenopus oocyte visualized by electron microscopy. Adapted from (Aebi et al. 1986).

The "lowest" organism in evolution identified so far containing both, A-and B-type lamins, is *D.melanogaster*. In mammals, three independent genes encode all nuclear lamins. A-type lamins, Lamin A and C, are encoded by a single gene that is alternatively spliced, the B-type Lamins, Lamin B1 and B2, are encoded by two individual genes (Broers *et al.* 1997; Foisner 2001). In addition to the major lamin variants, two minor isoforms of Lamin A and C are known: Lamin AΔ10 (Machiels *et al.* 1996) and Lamin C2 (Furukawa *et al.* 1994), as well as a minor splice isoform of Lamin B2, termed Lamin B3 (Furukawa and Hotta 1993). Lamin C2 and Lamin B3 were reported to be germ cell specific. The expression of at least one major B-type lamin is essential for cell survival (Vergnes *et al.* 2004), whereas A-type lamins seem to be dispensable for cell viability (Fong *et al.* 2006) and are expressed in differentiated cells only (Constantinescu *et al.* 2006; Rober *et al.* 1989). Despite the fact of the discovery of nuclear Actin and Actin-related proteins (ARPs) (Chen and Shen 2007; Goodson and Hawse

2002; McDonald *et al.* 2006; Pederson and Aebi 2005) and a nuclear form of Myosin I (Pestic-Dragovich *et al.* 2000), the nuclear lamina represents the only proteinaceous filament network within the nucleus known so far. Actin and Myosin I, both well known constituents of the filamentous cytoskeleton, were reported to exist in the nucleus in a different conformation as compared to the cytoplasm (Jockusch *et al.* 2006).

The proposed functions of the nuclear lamina can be grouped into two major activities. First, the supply of mechanical stability and structure to the nucleus preventing the physical collapse of the nuclear sphere, especially in tissues exposed to mechanical stress (for instance in skeletal muscle fibers) (Gotzmann and Foisner 2006; Houben *et al.* 2007; Lee *et al.* 2007) and second, a role in gene expression supported by the reported interaction of lamins with transcriptional regulators, such as pRB, chromatin and chromatin organizing components, such as Histones and BAF (Barrier-to-Autointegration Factor) (Bridger *et al.* 2007; Gotzmann and Foisner 2006; Gruenbaum *et al.* 2003). Both functions of the nuclear lamina can help to explain the diverse phenotypes observed in a highly heterogeneous group of inherited human diseases caused by mutations in the Lamin A/C gene or in integral membrane proteins binding to Lamins A/C (Gotzmann and Foisner 2006). This group of late-onset diseases is collectively termed "laminopathies" and includes such different phenotypes as muscular dystrophy, dilated cardiomyopathy, lipodystrophy, bone structure- and skin defects and most severely, a premature aging pathology (see also chapter 2.4). Besides their well-characterized filamentous structure at the nuclear periphery, nuclear lamins were also found in the nuclear interior, mostly in intranuclear foci (Gotzmann and Foisner 1999; Muralikrishna *et al.* 2001). Nucleoplasmic Lamins A/C, together with the nuclear protein LAP2α (Lamina-associated Polypeptide 2), was also shown to stabilize and thereby regulate the crucial cell cycle regulator and differentiation factor Retinoblastoma Protein (pRB) (Johnson *et al.* 2004; Pekovic *et al.* 2007). Clearly these findings corroborate the current notion, that Lamins A/C are not only needed for the mechanical stability of the nucleus and the structure of the nuclear envelope, but are actively involved in the regulation of cell cycle progression (Dechat *et al.* 2007; Dyer *et al.* 1997) and differentiation (Boguslavsky *et al.* 2006; Constantinescu *et al.* 2006).

Localisation	Assembly group	Chain type	Examples
Cytoplasm	1	I	Acidic keratins (pI ~ 4.9–5.7)
		II	Basic keratins (pI ~ 6.1–7.8)
	2	III	Vimentin, desmin, glial fibrillary acidic protein (GFAP), peripherin
		IV	Neurofilament proteins (NF-L, NF-M, NF-H), internexin
Cell nucleus	3	V	Lamins

Table 1: Classification of intermediate filaments in vertebrates. Adapted from (Strelkov et al. 2003).

2.4 Human Diseases associated with the Nuclear Envelope

During the last decades a multitude of severe, inherited human diseases were linked to mutations in several resident proteins of the nuclear envelope or lamina and collectively termed “Envelopathies” (Nagano and Arahata 2000; Somech *et al.* 2005a) or more specifically, “Laminopathies” (Worman and Bonne 2007). Envelopathies affect diverse somatic tissues, including skeletal and heart muscle, adipose tissue, the nervous system, skin, bone structure and blood cells, consequently causing a broad variety of cellular (Fig. 10) and clinical phenotypes (http://www.ncbi.nlm.nih.gov/OMIM) (Rankin and Ellard 2006). The most common feature of envelopathies is a late onset muscular dystrophy as observed in Emery-Dreifuss Muscular Dystrophy (EDMD) (Muchir and Worman 2007) and heart problems like dilated cardiomyopathy (DCM), but each patient shows an individual pattern of phenotypes, which vary significantly in severity.

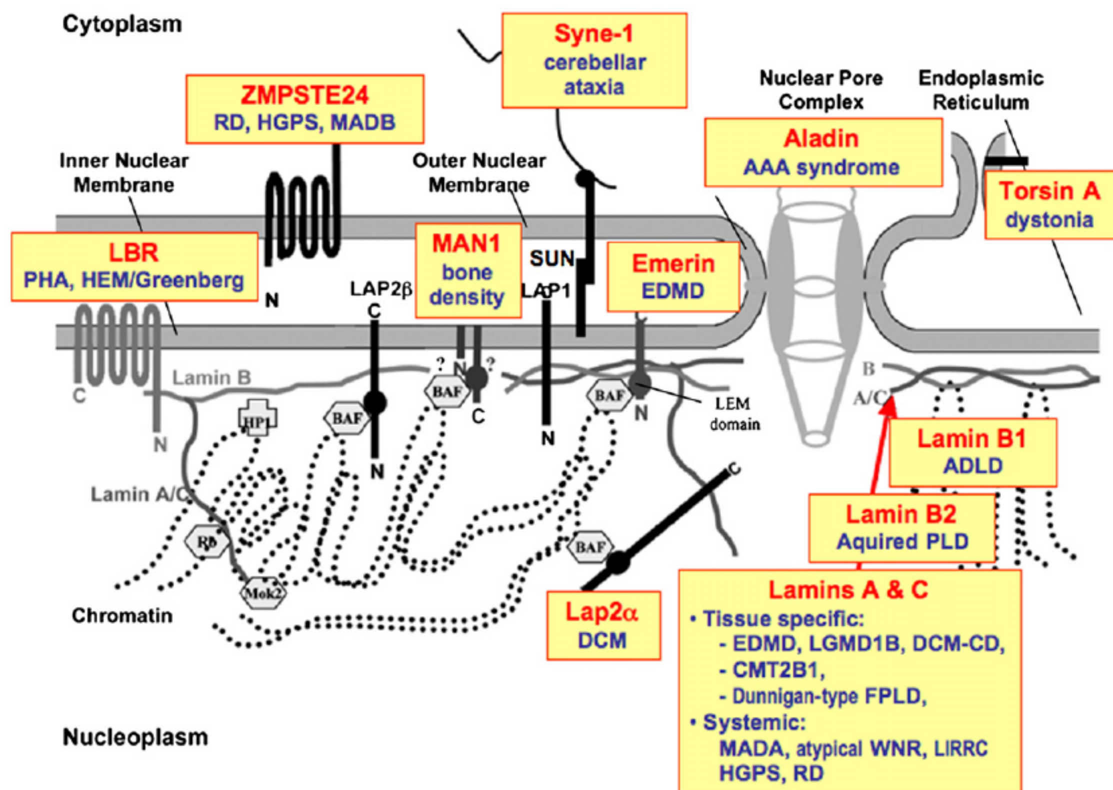


Figure 10: Summary of currently known gene defects linked to human diseases. Restrictive Dermopathy (RD), Mandibuloacral Dysplasia A/B (MADA/MADB), Pelger-Huet Anomaly (PHA), Adult-onset Autosomal dominant Leukodystrophy (ADLD), Limb-Girdle Muscular Dystrophy 1b (LGMD1b), Charcot-Marie-Tooth syndrome 2B1 (CMT2B1), Familial Partial Lipodystrophy (FPLD), Werner Syndrome (WNR). Adapted from (Worman and Bonne 2007).

Confusingly, the position of the mutation does not predict the prevalent disease phenotype of the patient in most cases. Even within a single affected family different family members carrying the

identical mutation, may exert divergent pathological phenotypes. So far, there is no conclusive explanation for this phenotypical plasticity. At the cellular level, abnormal nuclear shapes and altered gene expression profiles were reported (Gotzmann and Foisner 2006; Maraldi *et al.* 2005; Maraldi *et al.* 2006; Mounkes *et al.* 2003; Worman and Bonne 2007).

The most systemic pathology linked to Lamin A/C mutations known is the Hutchison-Gilford premature aging syndrome (HGPS), affecting essentially all tissues in the body of patients (Halaschek-Wiener and Brooks-Wilson 2007; Hennekam 2006; Kudlow *et al.* 2007).

The triggers for this severe disease are mutations in the LMNA gene leading to aberrant splicing of the Lamin A/C mRNA. This results in the accumulation of an unprocessable, stably farnesylated pre-Lamin A/C in the nucleus, which is thought to be the cause for the molecular pathologies of the disease. The molecular details why the pre-Lamin A accumulation leads to accelerated aging is still unknown. One of the most pressing questions in the field today is how mutations in the Lamin A/C gene can give rise to such a variety of disease phenotypes. Currently more than two hundred disease-causing mutations have been mapped all along the Lamin A/C gene (Fig. 11).

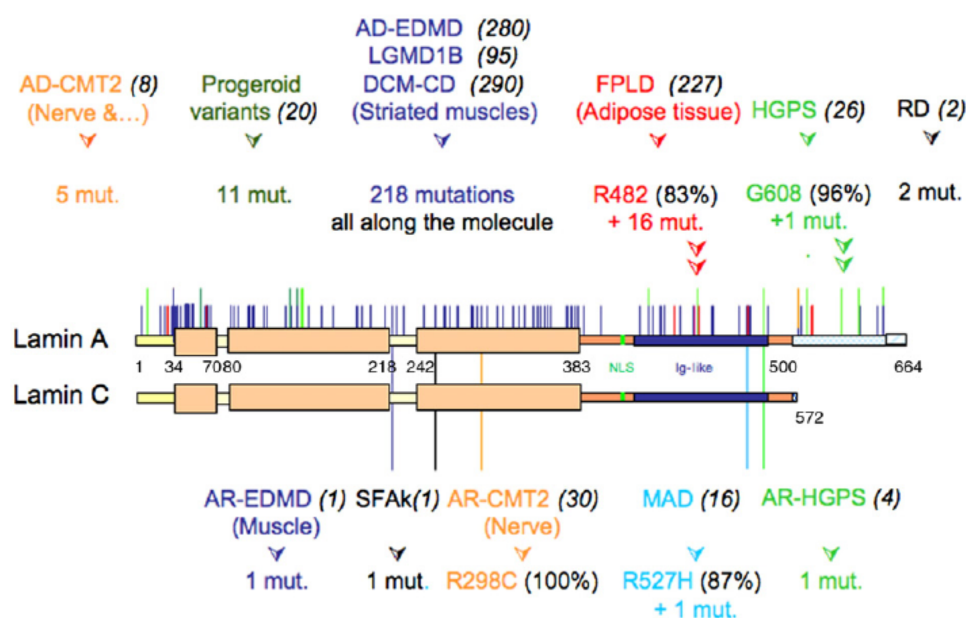


Figure 11: Identified mutations linked to inherited diseases in the Lamin A/C gene. Adapted from (Worman and Bonne 2007).

As most envelopathies have a late onset during childhood, one hypothesis is that these mutations specifically affect the function of somatic stem cells thereby impairing tissue regeneration (Gotzmann and Foisner 2004; 2006). Therefore, patients may show no obvious phenotype at birth, but health problems appear as soon as tissue regeneration is required.

Aside from mutations mapped within the Lamins A/C gene itself, also defects in genes encoding proteins involved in the posttranslational processing of Lamins A/C give rise to clinically similar

diseases. Both, Lamin A- and B-type lamins are C-terminally modified with a farnesyl group, but whereas B-type lamins are incorporated into the nuclear lamina as farnesylated proteins, A-type lamins undergo further processing. These maturation steps include two C-terminal cleavage steps by the enzyme Zmpste24. Deleterious mutations in the Zmpste24 gene cause an accumulation of premature Lamin A within the nucleus, which was also associated with HGPS.
These findings suggest that a tightly controlled balance between pre-and mature Lamin A/C in the nucleus is essential for viability. Interestingly this balance may also be important in the regular human aging process (Vlcek and Foisner 2007).
All together, the nuclear lamina and the proteins associated with the nuclear envelope exert pivotal regulatory functions in the nucleus – far beyond a simple structural role. With a very high probability, much more disease phenotypes will be linked to mutations in already known or in any of the 60 novel – so far not characterized – INM proteins in the future. One of the major aims in this field is the elucidation of the molecular pathways disturbed by disease-causing mutations, as this will enable clinical research to develop new strategies for therapies in the future.

2.5 LEM-Domain Containing Proteins at the Nuclear Periphery

The LEM protein family comprises several proteins that contain an about 40 aa long structural motif termed LEM (LAP2-Emerin-MAN1) (Cai *et al.* 2001; Laguri *et al.* 2001; Lin *et al.* 2000), which was found to interact with the DNA cross linking protein BAF ((Shumaker *et al.* 2001), see also chapter 2.6). The founding members of the family constitute several LAP2 isoforms, Emerin and MAN1 and database searches revealed the existence of other potential LEM domain containing proteins in the mammalian genomes (provisionally termed LEM2, 3, 4 and 5; (Lee and Wilson 2004)). While the LEM domain was initially described as a conserved stretch forming a helix-loop-helix motif (Cai *et al.* 2001; Laguri *et al.* 2001; Lin *et al.* 2000) (Fig. 12) in nuclear proteins, from an evolutionary point of view it may have evolved from an ancient helix-loop-helix motif functioning as a DNA binding domain. Later in evolution this domain developed into two separate motifs, namely the SAP domain (SAF A/B, Acinus, PIAS) (Fig. 13) and the actual LEM domain (Mans *et al.* 2004). Whereas the SAP domain still represents a motif which binds directly to DNA (Aravind and Koonin 2000), the LEM-domain establishes dynamic protein-chromatin complexes indirectly via interaction with a small, DNA-crosslinking molecule termed BAF (Barrier-to-Autointegration-Factor) (see also chapter 2.6) (Shumaker *et al.* 2001).

Most of the so far analyzed LEM proteins contain at least one transmembrane domain, reside at the INM (Wagner and Krohne 2007) and interact with the nuclear lamina. The only clearly reported exception among the otherwise membrane anchored LEM proteins is LAP2α, a specific LAP2 isoform lacking a transmembrane domain and localizing in the nucleoplasm.

A proposed major function of the nuclear lamina and its associated proteins is the maintenance of nuclear integrity and the organization of the peripheral chromatin (Fig. 14) (Sullivan *et al.* 1999; Vergnes *et al.* 2004; Verstraeten *et al.* 2007; Wagner and Krohne 2007). The nuclear periphery has previously been characterized as a nuclear region with increased levels of dense heterochromatin (Figure 15) and was therefore proposed to constitute a transcriptional silent region within the nucleus. Dense patches of peripheral heterochromatin are only interrupted by euchromatic areas at the NPCs (Akhtar and Gasser 2007).

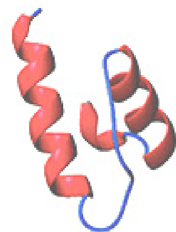


Figure 12: Structure of the LEM-domain. Adapted from (Cai et al. 2001).

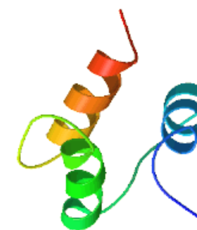


Figure 13: Structure of the SAP-domain. Adapted from the "Pfam" database (www.pfam.sanger.ac.uk)

There is increasing evidence that proteins at the INM and nuclear lamina (Figure 13) exert important regulatory functions also on the transcriptional activity of attached chromosomal regions (Bakay *et al.* 2006; Masny *et al.* 2004; Somech *et al.* 2005b; Verstraeten *et al.* 2007).

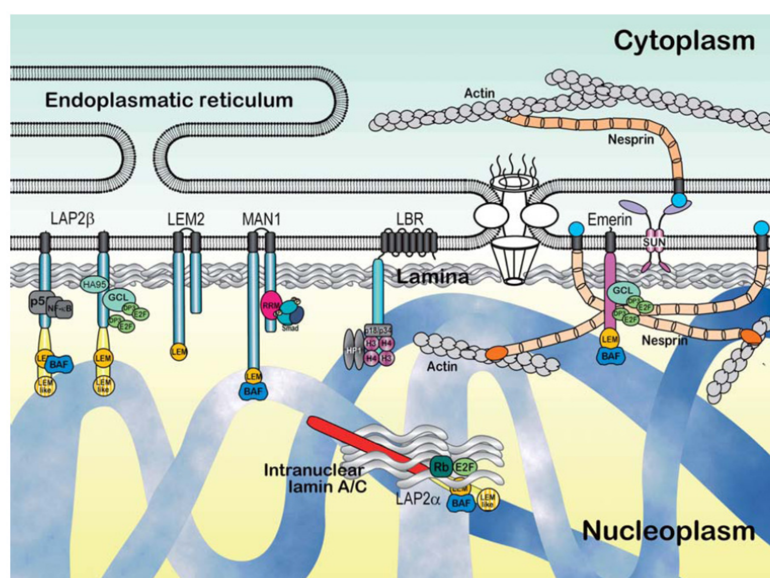


Figure 14: Schematic drawing of known inner nuclear membrane and their interactions at the nuclear periphery. Adapted from (Wagner and Krohne 2007).

Surprisingly, MAN1, a well known INM LEM protein, was found to be directly involved in TGFβ and BMP signaling via specific tethering of Smad proteins at the NE (Bengtsson 2007) and Emerin, another long known LEM protein at the INM, was reported to regulate β-Catenin activity (Markiewicz *et al.* 2006). These findings drew a lot of attention to the molecular analysis of INM components and this broad interest was even further enhanced by the discovery that a heterogeneous disease group, termed "envelopathies", is linked to mutations in some of these proteins. Recently, a proteomic screen performed by Schirmer and colleagues identified about 60 novel integral INM proteins in rat liver cells, which were termed NETs 1-62 (Nuclear Envelope Transmembrane) (Schirmer *et al.* 2003; 2005). About one third of the human NET-encoding genes have been mapped to known dystrophy-linked chromosomal regions, suggesting important roles of nuclear envelope proteins in regulatory functions of skeletal muscle physiology. Aside from diseases affecting muscle tissue, a broad diversity of disease phenotypes were linked to mutations in INM proteins or nuclear lamins (see also chapter 2.4) which suggests that these proteins may have particular tissue- or cell type specific functions.

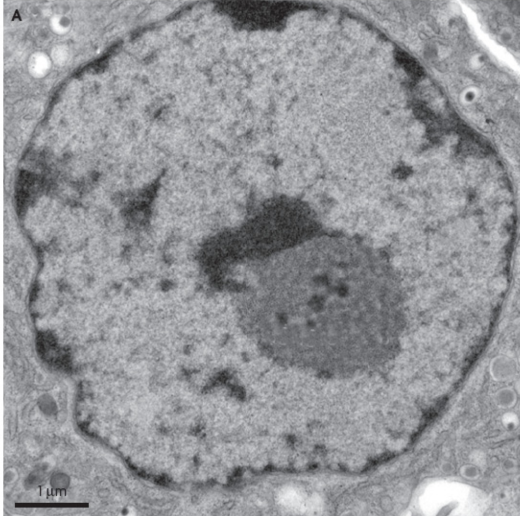


Figure 15: Electron micrograph of a mammalian cell nucleus. Adapted from (Akhtar and Gasser 2007)

The functional characterization of one novel protein identified by Schirmer et al., NET25, which was then found to be a Lamin A-interacting LEM domain containing protein and thus was renamed LEM2, was a major topic of this thesis.

2.6 Barrier-to-Autointegration Factor – a BAFling Little Protein

The following manuscript introduces the interaction partner of LEM domain containing proteins, BAF. This chapter gives a brief overview of the content of the review article and highlights additional important findings, which were not included in the review.

BAF (Barrier-to-Autointegration Factor) is a small, abundantly expressed, highly conserved protein, which was initially identified as a cellular component preventing the autointegration of retroviral DNA, and favors its integration into the host genome. It was then discovered, that BAF mediates the interaction of LEM proteins with chromatin. BAF itself binds to double-stranded DNA in a non-sequence specific way (Cai *et al.* 1998). A comprehensive structural study of the LEM-BAF complex revealed that a LEM domain/BAF/DNA complex could only be formed by a BAF dimer that binds two DNA strands and a single LEM domain. According to the authors, this configuration prevents the formation of mixed complexes with more than one LEM-protein (Cai *et al.* 2007). Besides the LEM domain, BAF interacts with multiple other nuclear components including histones H1.1 and H3 (Montes de Oca *et al.* 2005), lamins and various homeodomain transcription factors (Wang *et al.* 2002). BAF exerts functions in several cellular processes, such as chromatin organization and compaction, regulation of transcription, developmental functions and nuclear envelope assembly (Margalit *et al.* 2007a). Recently, BAF was identified as a regulator of cell fusion in C. elegans by inhibiting the expression of the *fusogen* protein eff-1 through specific binding to the eff-1 promoter (Margalit *et al.* 2007b).

So far, the intracellular localization of BAF was supposed to be primarily nuclear as all described BAF interaction partners are found there. Recently, Haraguchi and colleagues reported that BAF localization is dependent on the physiological state of cells and they claim that BAF is translocated from the nucleus to the cytoplasm upon transition to senescence. In contrast, immortal cell lines also exhibited high levels of cytoplasmic BAF, leading to the speculation that the nuclear fraction of BAF suppresses cellular senescence as well as cancer progression in non-transformed cells. Furthermore, nuclear BAF was found to be associated with S-phase progression in both mortal and immortal cells (Haraguchi *et al.* 2007). In addition, a highly related protein termed BAF-like has recently been reported, which forms homo- and heterodimers with BAF *in vitro.* Interestingly, the BAF-like homodimer showed no significant binding affinity to DNA and both the homo- and heterodimer did not bind to the LEM domain *in vitro*, corroborating the finding of Cai and colleagues (Cai *et al.* 2007) that only a BAF homodimer is able to form a complex with DNA and the LEM domain. Consequently, it was proposed that the BAF-like protein might function as a modulator of BAF interactions (Tifft *et al.* 2006).

Review TRENDS in Cell Biology Vol.17 No.4
ELSEVIER
Full text provided by www.sciencedirect.com ScienceDirect

Barrier-to-autointegration factor – a BAFfling little protein

Ayelet Margalit[1], Andreas Brachner[2], Josef Gotzmann[2], Roland Foisner[2] and Yosef Gruenbaum[1]

[1] Department of Genetics, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[2] Max F. Perutz Laboratories, Medical University of Vienna, A-1030 Vienna, Austria

Barrier-to-autointegration factor (BAF) is an abundant, highly conserved, small and essential protein that binds to dsDNA, chromatin, nuclear lamina proteins, histones and various transcription factors. It was discovered as a cellular component of retrovirus pre-integration complex that inhibits their autointegration *in vitro*. BAF is also required for many cellular functions, including the higher-order organization of chromatin and the transcription of specific genes. Recent findings suggest further roles for BAF, including nuclear envelope assembly, regulating specific developmental processes and regulating retrovirus infectivity. At least some of these roles are controlled by phosphorylation of the BAF N-terminus by the vaccinia-related kinase. Here, we give an overview of recent advances in the field of BAF with special emphasis on evolution, interacting partners and functions.

Barrier-to-autointegration factor

BAF is a 10 kDa evolutionary conserved protein (Figure 1). There are at least six *BAF* homologous genes in the mammalian genome including *BAF*, *BAF-Like* (*BAF-L*) (Box 1) and four more genes that are related to *BAF* [1]. Of this latter group, it is currently unknown which are active genes. BAF was first discovered by its ability to prevent autointegration of the Moloney Murine Leukemia Virus MoMLV and, hence, was named barrier-to-autointegration factor (BAF) [2]. BAF is a cellular component of viral pre-integration complexes formed in the cytoplasm of cells infected by HIV-1 or MoMLV viruses [3]. BAF is also localized in the nucleoplasm and is highly enriched at the nuclear envelope [4–7] (Box 2). Cytoplasmic and nuclear BAF pools are highly dynamic, but fail to replenish each other, suggesting a tight regulation of BAF subcellular localization [4].

BAF has many binding partners (Table 1). The best characterized interacting motif is the Lamina-associated polypeptide 2 (LAP2)–Emerin–MAN1 (LEM) domain [8] (Box 3), found in the LEM protein family. Most LEM domain proteins are integral to the inner nuclear membrane, form complexes with nuclear lamins and are involved in many of the nuclear envelope functions. Direct interactions between BAF and LEM proteins were confirmed by NMR chemical shift and biochemical analyses [9,10] including that of LAP2α and LAP2β [11], emerin [12,13], MAN1 [14] and LEM-2 [15]. A second unrelated BAF-binding region was observed in MAN1 [14] and LEM-2 [16]. The binding of LEM proteins to BAF regulates retrovirus infection [2,17], nuclear assembly [5,7], cell cycle progression [6], developmental processes [6,7] and even gene expression [1]. Here, we summarize new findings on BAF cellular functions and BAF regulation by binding partners.

BAF is required for the biogenesis of retroviruses

BAF has a key role in the biogenesis of MoMLV and HIV-1 retroviruses. At the early phases of infection, when the retrovirus enters the cell cytoplasm, the reverse transcription complex (RTC) reverse-transcribes the viral RNA genome. The newly transcribed dsDNA remains in the cytoplasm as a nucleoprotein pre-integration complex (PIC). The presence of BAF in PICs from MoMLV-infected cells blocks autointegration of the viral DNA *in vitro*, whereas removal of BAF from these complexes with high salt caused self-destruction of the virus by autointegration of the viral DNA [2,3,17,18]. The recruitment of BAF to PICs probably occurs during the initial stages of PIC formation. The current model suggests that BAF-bridging of DNA protects the viral DNA from autointegration catalyzed by its own integrase, which is achieved by compacting and making DNA inaccessible for integration. Supporting this model is the ability of BAF to cross-bridge and compact viral DNA when reconstituted with salt-stripped MoMLV PICs [18]. BAF dimers bind to DNA in a non-sequence-specific manner, with subsequent protein dimerization or oligomerization governed by the length of the DNA molecule to which it binds. The binding of BAF to 7-bp dsDNA molecules was shown to form complexes containing a single BAF dimer cross-bridging two DNA molecules [19], whereas the binding to 21-bp dsDNA molecules led to the formation of complexes containing six BAF dimers and six DNA molecules [20]. When binding to DNA, BAF undergoes conformational changes [21]. This binding is probably regulated by BAF phosphorylation, because phosphorylation of the BAF N-terminus dramatically reduced its ability to interact with DNA [22].

The atomic structure of the BAF dimer was determined to a 1.9-Å resolution both by NMR spectroscopy and X-ray crystallography [23,24]. Each monomer in the dimer is made of two helix-hairpin-helix motifs that bind to DNA [24]. The crystallographic analysis of BAF bound to DNA

Corresponding author: Gruenbaum, Y. (gru@vms.huji.ac.il).
Available online 21 February 2007.

also confirmed its ability to cross-bridge DNA molecules in solution by helix-hairpin-helix motifs located on opposite surfaces of the dimer [23]. This structure suggests a simple model in which BAF induces DNA condensation by cross-linking distant regions of DNA [19].

In addition to regulating autointegration of MoMLV, BAF is also able to promote the association of MoMLV and HIV-1 PICs with host cellular DNA [18]. In dividing HeLa cells and non-dividing primary macrophages, the downregulation of BAF, or BAF-interacting LEM proteins: LAP2α or emerin, but not MAN1, significantly enhances cell resistance to HIV-1 infection [25]. BAF and LAP2α, but not emerin or MAN1, are also required for MoMLV infectivity. This demonstrates the involvement of a specific LEM protein in retrovirus infection [25,26]. Biochemical analysis showed that, although BAF and emerin are not required for nuclear translocation of PICs [25], they are required for the localization of viral cDNA and proteins (such as integrase) [25] in the nucleus, probably through the direct binding of the viral integrase to BAF [3]. By contrast, other studies [27] found that levels of HIV-1 and MoMLV infection in HeLa-P4 cells following the downregulation of emerin, BAF or LAP2α, and in knockout mouse embryo fibroblasts lacking *EMR1* or *LMNA* (in which emerin is mislocalized) were similar to that in control cells. These contrasting results might be due to different clones of the cell lines and the exact experimental conditions.

BAF-binding to the structural components of the HIV-1 virions, p55 Gag and Matrix (p17), suggest additional roles for BAF in HIV-1 biogenesis [28]. Further studies are required to define the roles of BAF, emerin and LAP2α in retrovirus biogenesis *in vivo*.

BAF regulates nuclear assembly and organization

An important event during cell division is the accurate reassembly of the nuclear envelope during the last stages of mitosis. It is based on a precise sequence of events including the targeting of specific proteins to chromosomal surfaces, membrane recruitment and fusion, assembly of nuclear pore complexes (NPCs) and transport of the bulk of lamins into the nucleus to form the nuclear lamina [29]. Lamins, LEM proteins and BAF have essential roles in the process of nuclear envelope assembly [7,30]. Fluorescence Recovery After Photobleaching (FRAP) analysis showed that nuclear and cytoplasmic GFP–BAF are both highly motile during interphase (see above) [4], suggesting numerous transient interactions of BAF with stably anchored proteins, such as LEM proteins, core histone H3 and selected linker histone H1.1 [31] (Table 1). FRAP analysis also demonstrated that BAF in the mitotic nucleus forms relatively stable complexes on chromosomes. During anaphase, a fraction of BAF associates with LAP2α at the telomere regions and is stabilized by LAP2α homo-oligomerization and recruitment of LEM-domain containing membrane proteins, such as emerin and LAP2β [32]. During nuclear assembly, the growing LAP2α–BAF complexes at the telomeres spread to ‘core regions’ on the surface of the decondensing chromosomes [5,7,30,32]. The accumulation of BAF in the core regions might induce a spatially regulated DNA condensation mediated by the DNA cross-linking activity of BAF and, thus, might contribute to chromatin reorganization. In addition, these core regions probably serve as a nucleation site for nuclear membrane assembly by recruiting proteins of the LEM-domain family.

Overexpressed Myc-tagged BAF in HeLa cells was shown to be localized at mitotic chromosomes and to cause the redistribution of LAP2α from the cytoplasm to chromosomal surfaces (Figure 2); the localization of LAP2β and emerin, however, was not affected (A.G. and R.F., unpublished observations). Expression of mutated BAF, incapable of binding to DNA, blocked the recruitment of emerin and LAP2β to core regions and inhibited the assembly of these proteins and of lamin A into the nuclear envelope, although lamin B was not affected [5]. Likewise, mutated emerin, incapable of binding to BAF, failed to localize at the nuclear envelope. In conclusion, LEM proteins show a differential binding to BAF at defined stages of the cell cycle and these interactions are probably required for BAF roles in normal cell cycle progression.

The function of BAF in nuclear assembly and chromatin organization was also tested in *Xenopus* egg nuclear assembly extracts. A subtle increase in BAF concentration enhanced nuclear assembly whereas larger amounts of excess BAF caused massive DNA compaction and inhibited this process [33]. This suggests that small amounts of excess BAF are properly regulated and have a positive role in nuclear envelope assembly. Determining the way by which BAF expression is regulated is a challenge for future studies.

The function of BAF in nuclear assembly and chromatin organization was also tested in the context of living animals using *Caenorhabditis elegans* and *Drosophila*. Having only one copy of BAF in *Drosophila* or an extra copy of functional BAF in *C. elegans* had no effect on nuclear morphology or development [6,30], therefore suggesting that, in contrast to nuclear assembly in *Xenopus* extracts, *C. elegans* and *Drosophila* can tolerate excess or reduced amounts of BAF.

RNA interference-mediated BAF knockdown in *C. elegans* caused death before the 100-cell stage and revealed BAF as an essential gene [7]. At the cellular level, depletion of BAF led to anaphase-bridged chromatin and defects in the nuclear envelope with mislocalized lamin and inner nuclear membrane (INM) proteins: emerin and MAN1 (LEM-2) [7]. Similarly, loss of lamins or simultaneous knockdown of emerin and LEM-2 in *C. elegans* showed defects in chromosome segregation and nuclear envelope formation [15,16]. Thus, the assembly of BAF, LEM proteins, and lamins at the chromosomes during cell division is mutually dependent and is required for proper chromosome segregation and cell cycle progression [34]. In *C. elegans*, BAF's functions in nuclear assembly seem to be independent and precede its roles in chromatin organization, indicating that the nuclear assembly defects are not necessarily the direct result of defects in chromatin organization [30]. Also, BAF-null *Drosophila* mutants showed lethality through mitotic defects and a profound reorganization of chromatin in interphase nuclei [6], supporting BAF roles in both chromatin organization and nuclear assembly.

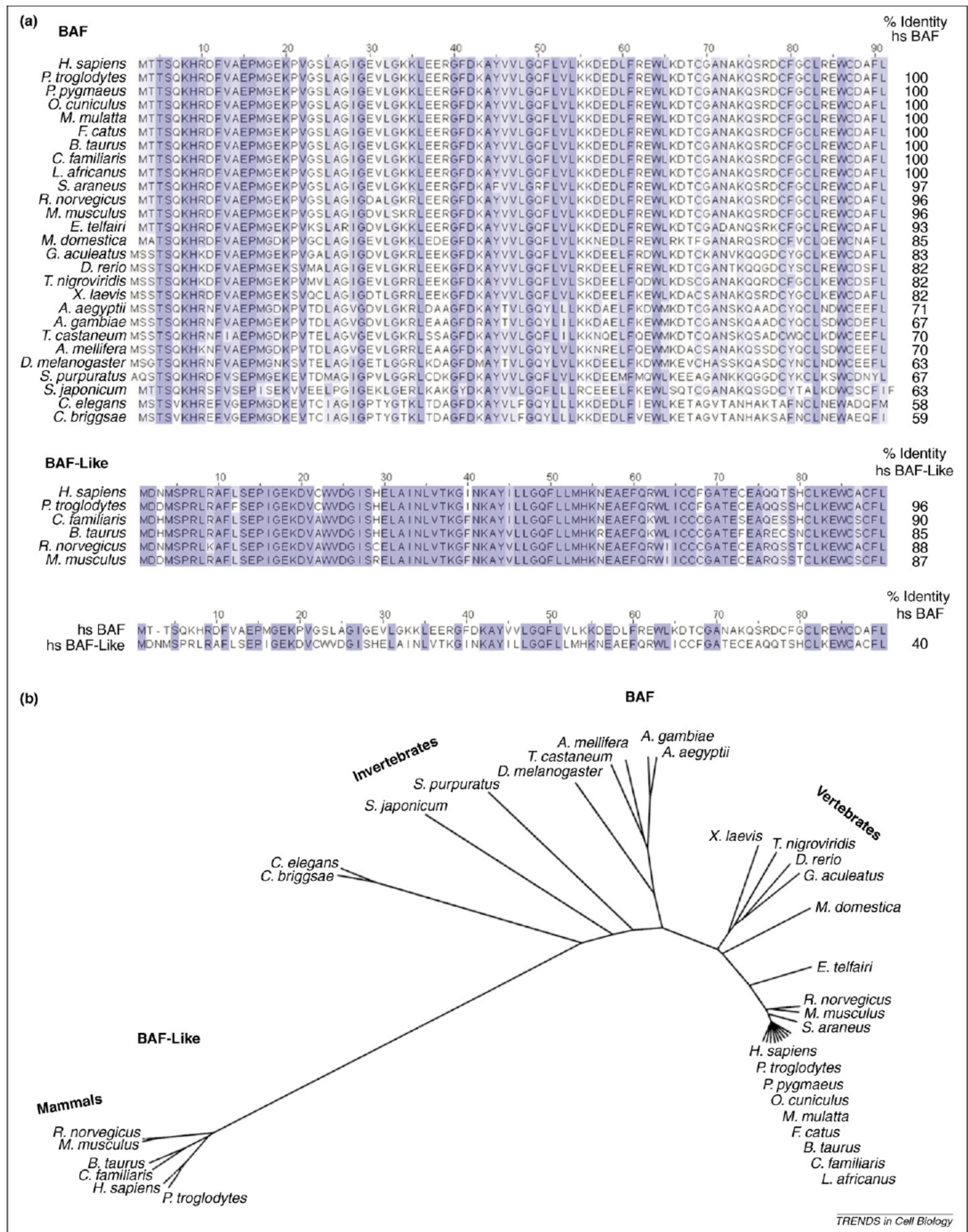


Figure 1. BAF is an evolutionary conserved protein. **(a)** Multiple sequence alignment of BAF and BAF-L primary sequences along with the resulting phylogenetic tree analysis. Multiple sequence alignment was performed using the software ClustalW (http://www.ebi.ac.uk/clustalw/index.html) and a Blosum 62 matrix. High consensus scores (>80% identity) are in dark blue, and mid-consensus (60%) and low-consensus scores (>40%) are in mid blue and light blue, respectively. **(b)** The unrooted

Box 1. BAF-Like protein regulates BAF activity

The BAF-L and BAF proteins are 40% identical and 53% similar [41]. Homologs to human BAF-Like (hBAF-L) with a high degree of identity (~90%) are present in other mammals, but not in other vertebrates or invertebrates (see Figure 1 in the main text). Comparing hBAF-L and hBAF reveals two highly conserved regions containing amino acids 9 to 19 and 39 to 57. These regions are involved in BAF dimerization and are outside the DNA-binding region of BAF [41]. Indeed, BAF-L can form homodimers and heterodimers with BAF, but cannot bind dsDNA *in vitro* [41]. Moreover, BAF–BAF-L heterodimers, but not BAF-L homodimers, can bind LEM proteins *in vitro*. These observations led to the intriguing model in which BAF-L regulates the function of BAF. According to this model, BAF–BAF-L heterodimers, which contain only one DNA binding domain, cannot cross-bridge DNA and therefore counteract the DNA compaction activity of BAF homodimers [41].

BAF-L has a tissue-restricted expression, being highest in pancreas and testis and absent in heart, skeletal muscle and kidney [41]. The high expression of BAF-L in the testis could be connected to the fact that the mammalian GCL is also highly expressed in testis [44]. This raises the interesting possibility that BAF-L in the testis regulates the competition of BAF with GCL for binding to LEM proteins and affects LEM domain protein functions in the germline.

Box 2. The nuclear envelope and laminopathies

The nuclear envelope surrounds the nucleus and separates nuclear and cytoplasmic activities. It is composed of outer and inner nuclear membranes that are joined at the nuclear pore complexes (NPCs). The NPCs mediate transport of macromolecules between the cytoplasm and the nucleoplasm. The nuclear lamina is positioned between the INM and the peripheral chromatin. It is a protein meshwork composed of lamins, which are intermediate filament proteins, and lamin-associated proteins [45]. In humans, there are three lamin genes encoding A- and B-type lamins [46]. Many lamin-associated proteins are integral proteins of the INM. Some of the INM proteins share common domains including the LEM-domain [47] (Box 3). Mutations in both A-type or B-type lamins, in addition to mutations in lamin-associated proteins, cause a heterogeneous group of heritable human diseases that are collectively termed 'laminopathies' [48,49]. At least 13 different laminopathies have already been described ranging from muscular dystrophies to accelerated aging; however, the molecular mechanisms of these diseases are poorly understood [50]. The conserved LEM-domain–BAF interaction is particularly interesting, as mutations in several LEM proteins, such as emerin, MAN1 and LAP2α have been linked to laminopathies [51]. It is conceivable that mutations in lamins or emerin affect the lamin–emerin–BAF complex and its functions in chromatin organization and gene expression. Interestingly, BAF also seems to be able to bind directly to A-type lamins, although with lower affinity than to the LEM proteins [40].

BAF regulates gene expression

BAF regulates gene expression at several levels as outlined below, which are not mutually exclusive. Heterochromatin formation is regulated by chromatin-modifying complexes and epigenetic mechanisms involving modifications of histone tails [35]. BAF is involved in heterochromatin formation, probably by both its DNA-bridging activity and its ability to bind to the tail region of core histone H3 [31]. The latter raises the intriguing possibility that BAF might contribute to the epigenetic regulation of chromatin. Chromatin immunoprecipitation (ChiP) and direct binding of BAF to modified histone H3 tails would help determine BAF's involvement in epigenetic chromatin regulation.

In addition, BAF interacts *in vitro* with the murine retinal cone-rod homeobox factor (Crx) and is able to repress CRX-dependent reporter activity *in vivo* [1]. Although the molecular mechanism of BAF-mediated repression is unknown, it might be linked to its DNA-bridging and condensation activity. This scenario is highly intriguing because it describes a generic and 'unspecific' mode of action of BAF as a cofactor in gene repression. According to this model, specificity and regulation of BAF-mediated gene repression is achieved through the binding of BAF to specific transcription factors, which mediates its targeting to respective promoter sequences. In this model, BAF regulates specific gene expression through its interaction with LEM proteins, many of which interact directly with transcriptional regulators. For instance, LAP2β binds to the transcription repressor germ cell-less (GCL), which, in turn, binds to E2F–DP transcription factor heterotrimers and inhibits E2F activity [36]. LAP2α binds to the retinoblastoma protein pRB, with which it cooperates in repressing E2F activity [37]. MAN1 binds to Smad proteins and antagonizes Smad-dependent transcription [38] whereas emerin binds to β-catenin and regulates β-catenin/LEF transcription activity [39]. It was suggested that the interaction of INM LEM proteins with these transcriptional regulators antagonizes their transcriptional activity by recruiting them to the nuclear envelope. The presence of BAF at, or near, the LEM protein complexes could contribute to gene repression through its DNA-bridging activity. Another possibility is that BAF competes with these transcription factors for the binding of LEM proteins. For example, BAF competes with GCL for the binding of emerin [40].

BAF regulates key developmental processes

As a transcription regulator it is not surprising that BAF regulates key developmental processes. The best examples of BAF roles in development come from studies in *Drosophila* and *C. elegans*. Downregulation of BAF by RNA interference in *C. elegans* causes embryonic lethality; however, the inefficient downregulation of the *C. elegans* BAF enabled worms to survive embryogenesis and grow into sterile adults with misplaced gonads. Other tissue-specific phenotypes were also noticed [7]. The misplacement of the gonads was due to a defect in the migration of the distal tip cells (DTCs), which guide the position of the developing gonads. A mild reduction of BAF expression probably affected the expression of genes that regulate DTC migration. Flies homozygous for a deletion in the *Drosophila BAF* gene (*baf*/*baf*) died at the larva-pupal transition [6]. The *baf*/*baf* homozygous flies had a small brain, missing imaginal discs and defects in cell proliferation and differentiation in the thoracic ganglia and the brain hemispheres [6]. The absence of phenotypes during the early stages of development was probably due to sufficient maternal BAF. Nuclei from the brain tissue were

phylogenetic tree was calculated from the alignment above using the Phylodendron software (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). Numbers on the right denote % identity to human sequences. Abbreviation: hs, *Homo sapiens*.

Table 1. BAF-interacting partners at different cell compartments

Localization	Binding partners	Refs
Cytoplasm	Components of the preintegration complex of MoMLV and HIV-1	[3,18]
Nuclear envelope	INM LEM proteins such as LAP2α, LAP2β, emerin, MAN1 and LEM-2	[14,54]
Nucleoplasm	Histones: histone H3 and linker histones	[31]
	Nuclear LEM domain proteins such as LAP2α	[32]
	Cone-Rod homeobox (CRX) and related homeodomain transcription activators	[1]
	dsDNA in a sequence-independent matter	[23]
Not determined	BAF-L	[41]
	Lamin A	[40]
	Vaccinia-related kinase (VRK)	[22,30,42]

lobulated, and had chromatin clumps, convolutions and defects during mitosis [6], suggesting that these phenotypes result from the loss of BAF function in cell cycle progression and nuclear organization.

The presence of BAF in most, but not all, human tissues, indicates its involvement in specific developmental processes. Interestingly, BAF is absent in the thymus and peripheral blood leukocytes, which are target cells for HIV-1 infection [28]. Although this paradox is still unexplained, it is worth noting that BAF is present in primary macrophages, which are also target cells for HIV-1 infection [25]. In the retina photoreceptor cells, BAF interacts with the homeodomain protein Crx, which is required for photoreceptor development and function [1]. Overexpression of BAF represses Crx activation and its binding to Crx is affected by a specific Crx mutation associated with photoreceptor dystrophy, suggesting that BAF is directly involved in photoreceptor development and integrity [1]. BAF is also expressed in skeletal muscle and heart [1,28], which are both affected in laminopathic diseases caused by BAF's interacting partners: emerin, LAP2α and lamin A. It is therefore tempting to suggest that mutations in BAF also lead to muscle laminopathies by disrupting its complexes with lamins and LEM proteins that regulate muscle gene expression.

Box 3. LEM proteins

The LEM domain is named after three mammalian members of this protein family: Lamina-associated polypeptide (LAP2), Emerin and MAN1 [47], and is composed of a 40 amino acid-long motif of two helices linked by a short turn [9,52]. Other members of the mammalian LEM protein family include LEM2 [53] and three uncharacterized proteins: LEM3, LEM4 and LEM5 [54]. *Drosophila* LEM proteins include otefin (the first characterized LEM protein) [55], Bocksbeutel and MAN1 [56], and *C. elegans* LEM proteins include Ce-emerin, LEM-2 and LEM-3 [57]. Most LEM proteins are integral membrane proteins of the INM; they bind to lamins at the nuclear envelope, forming a peripheral nuclear lamina scaffold [45,58–60]. The complexity of the mammalian LEM proteins further increases due to the expression of many alternatively spliced isoforms. For example, the *LAP2* gene encodes six isoforms, five of which (*LAP2β*, *LAP2ε*, *LAP2δ*, *LAP2γ*, and *LAP2ζ*) are closely related and, with the exception of *LAP2ζ*, are INM proteins [61]. LAP2α shares only the N-terminus containing the LEM domain with the other isoforms, lacks a transmembrane domain and localizes at the nucleoplasm [11]. The conserved interaction between BAF and LEM proteins suggests that they coevolved. Whereas BAF orthologs are absent from the *Saccharomyces cerevisiae* genome, there are two LEM2–MAN1-related genes: *HEH1* (formally called *SRC1*) and *HEH2* [62,63]. The gene products Heh1 and Heh2 are localized at the INM, similar to MAN1, and at least Heh1 binds directly to DNA. Therefore, with the evolvement of BAF complexes, their binding to chromatin has become more complex and regulated at more levels.

Posttranslational modifications regulate BAF protein interactions

The highly dynamic features of BAF during the cell cycle and the numerous BAF interactions suggest that the protein is tightly regulated. The molecular mechanisms of BAF regulation are just beginning to be discovered. Besides its regulation by heterodimerization with BAF-L [41] (Box 1), BAF undergoes cell cycle-specific phosphorylation on Ser4, Thr2 and Thr3 [22,42]. During mitosis, BAF is phosphorylated by the vaccinia-related kinase (VRK), a member of the casein kinase family [22]. The hyperphosphorylation of BAF causes the loss of DNA-binding, reduced emerin-binding and its redistribution to the cytoplasm [22,42]. BAF phosphorylation by VRK is conserved in *C. elegans* where VRK is localized at the nuclear envelope before its disassembly [30]. Moreover, VRK depletion prevents the disassociation of BAF and LEM proteins from mitotic chromosomes and causes abnormal nuclear envelope assembly [30], implying that

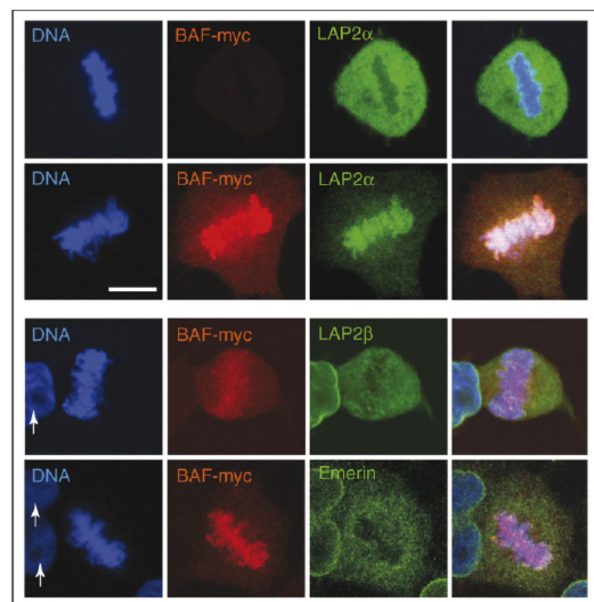


Figure 2. Different LEM proteins interact differently with BAF in metaphase. Non-transfected HeLa cells (first row) or cells stably expressing BAF-myc (lower 3 rows) were processed for immunofluorescence microscopy using antibodies against the Myc-tag (red) and against LAP2α, LAP2β or emerin (as indicated; green). DNA was stained with Hoechst dye (blue). Confocal images of metaphase and interphase cells in pseudocolor are shown. Arrows in blue images indicate interphase cells. The right panels show merged images. The scale bar represents 5 μm. Note that BAF-myc staining was much weaker in interphase versus metaphase cells, resulting in a barely detectable BAF-myc signal in interphase nuclei in the images shown.

the phosphorylation-dependent release of BAF from the chromatin during mitosis is essential for nuclear envelope assembly.

Phosphorylation of LEM proteins can lower their binding affinity to BAF. *In vitro* phosphorylation of emerin on five of its residues by mitotic *Xenopus* egg cytosol induces its dissociation from BAF–DNA complexes [43]. Unexpectedly, among the five phosphorylated residues, serine 175, which is located outside the LEM domain, is particularly important; indeed, a S175A mutation in emerin was shown to prevent the phosphorylation-dependent dissociation of BAF. The regulation of BAF–LEM proteins interaction must be temporally coordinated by phosphorylation, given the different kinetics of assembly of LEM proteins after mitosis.

Concluding remarks

Since the initial description of BAF ~9 years ago, BAF was found to be involved in many cellular and developmental functions and to have many partners (Table 1). There are still many open questions: how do BAF modifications regulate its localization and functions *in vivo*? How does BAF regulate gene expression and chromatin organization *in vivo*? What are the functions of BAF–LAP2α at the core regions? What are BAF's specific roles in development? Which of the other BAF homologs are functional? And, finally, what are the specific roles of BAF during retrovirus infection *in vivo?*

Conditional knockout mice for BAF and BAF-L will probably be a good strategy to start solving this puzzle. They will help determine the developmental functions of both BAF and BAF-L, in addition to the mechanism(s) enabling BAF-L to regulate BAF. The analysis of BAF in cells derived from laminopathic patients is also required to find potential links of BAF to diseases. It will also be as important to continue to follow this baffling gene in simpler model organisms including *C. elegans* and *Drosophila*, which have already been proven essential for the analysis of BAF functions.

Acknowledgements

We thank Andreas Gajewski, for providing Figure 2 and Shai Melcer for comments. Work in our laboratories was supported by grants from the EURO-Laminopathies research project of the European Commission (Contract LSHM-CT-2005–018690) to Y.G. and R.F., the Austrian Science Research Fund (FWF P17871) to R.F., and the USA–Israel Binational Foundation (BSF) and the Israel Science Foundation (ISF) to Y.G.

References

1 Wang, X. *et al.* (2002) Barrier to autointegration factor interacts with the cone-rod homeobox and represses its transactivation function. *J. Biol. Chem.* 277, 43288–43300
2 Lee, M.S. and Craigie, R. (1998) A previously unidentified host protein protects retroviral DNA from autointegration. *Proc. Natl. Acad. Sci. U.S.A.* 95, 1528–1533
3 Lin, C.W. and Engelman, A. (2003) The barrier-to-autointegration factor is a component of functional human immunodeficiency virus type 1 preintegration complexes. *J. Virol.* 77, 5030–5036
4 Shimi, T. *et al.* (2004) Dynamic interaction between BAF and emerin revealed by FRAP, FLIP, and FRET analyses in living HeLa cells. *J. Struct. Biol.* 147, 31–41
5 Haraguchi, T. *et al.* (2001) BAF is required for emerin assembly into the reforming nuclear envelope. *J. Cell Sci.* 114, 4575–4585
6 Furukawa, K. *et al.* (2003) Barrier-to-autointegration factor plays crucial roles in cell cycle progression and nuclear organization in *Drosophila*. *J. Cell Sci.* 116, 3811–3823
7 Margalit, A. *et al.* (2005) Barrier-to-autointegration factor is required to segregate and enclose chromosomes within the nuclear envelope and assemble the nuclear lamina. *Proc. Natl. Acad. Sci. U. S. A.* 102, 3290–3295
8 Furukawa, K. (1999) LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2–chromatin interaction. *J. Cell Sci.* 112, 2485–2492
9 Cai, M. *et al.* (2001) Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structures: one binds BAF and the other binds DNA. *EMBO J.* 20, 4399–4407
10 Shumaker, D.K. *et al.* (2001) LAP2 binds to BAF–DNA complexes: requirement for the LEM domain and modulation by variable regions. *EMBO J.* 20, 1754–1764
11 Dechat, T. *et al.* (2000) Lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J. Struct. Biol.* 129, 335–345
12 Lee, K.K. *et al.* (2001) Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. *J. Cell Sci.* 114, 4567–4573
13 Gruenbaum, Y. *et al.* (2002) The expression, lamin-dependent localization and RNAi depletion phenotype for emerin in *C. elegans*. *J. Cell Sci.* 115, 923–929
14 Mansharamani, M. and Wilson, K.L. (2005) Direct binding of nuclear membrane protein MAN1 to emerin *in vitro* and two modes of binding to barrier-to-autointegration factor. *J. Biol. Chem.* 280, 13863–13870
15 Liu, J. *et al.* (2000) Essential roles for *Caenorhabditis elegans* lamin gene in nuclear organization, cell cycle progression, and spatial organization of nuclear pore complexes. *Mol. Biol. Cell* 11, 3937–3947
16 Liu, J. *et al.* (2003) MAN1 and emerin have overlapping function(s) essential for chromosome segregation and cell division in *C. elegans*. *Proc. Natl. Acad. Sci. U. S. A.* 100, 4598–4603
17 Chen, H. and Engelman, A. (1998) The barrier-to-autointegration protein is a host factor for HIV type 1 integration. *Proc. Natl. Acad. Sci. U. S. A.* 95, 15270–15274
18 Suzuki, Y. and Craigie, R. (2002) Regulatory mechanisms by which barrier-to-autointegration factor blocks autointegration and stimulates intermolecular integration of Moloney murine leukemia virus preintegration complexes. *J. Virol.* 76, 12376–12380
19 Bradley, C.M. *et al.* (2005) Structural basis for DNA-bridging by barrier-to-autointegration factor. *Nat. Struct. Mol. Biol.* 12, 935–936
20 Zheng, R. *et al.* (2000) Barrier-to-autointegration factor (BAF) bridges DNA in a discrete, higher-order nucleoprotein complex. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8997–9002
21 Forne, I. *et al.* (2003) Identification of the autoantigen HB as the barrier-to-autointegration factor. *J. Biol. Chem.* 278, 50641–50644
22 Nichols, R.J. *et al.* (2006) The vaccinia-related kinases phosphorylate the N′ terminus of BAF, regulating its interaction with DNA and its retention in the nucleus. *Mol. Biol. Cell* 17, 2451–2464
23 Cai, M. *et al.* (1998) Solution structure of the cellular factor BAF responsible for protecting retroviral DNA from autointegration. *Nat. Struct. Biol.* 5, 903–909
24 Umland, T.C. *et al.* (2000) Structural basis of DNA-bridging by barrier-to-autointegration factor. *Biochemistry* 39, 9130–9138
25 Jacque, J.M. and Stevenson, M. (2006) The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. *Nature* 441, 581–582
26 Suzuki, Y. *et al.* (2004) LAP2α and BAF collaborate to organize the Moloney murine leukemia virus preintegration complex. *EMBO J.* 23, 4670–4678
27 Shun, M.C. *et al.* (2007) Wild-type levels of human immunodeficiency virus type 1 infectivity in the absence of cellular emerin protein. *J. Virol.* 81, 166–172
28 Mansharamani, M. *et al.* (2003) Barrier-to-autointegration factor BAF binds p55 Gag and matrix and is a host component of human immunodeficiency virus type 1 virions. *J. Virol.* 77, 13084–13092
29 Margalit, A. *et al.* (2005) The breaking and making of the nuclear envelope. *J. Cell. Biochem.* 95, 454–465
30 Gorjánácz, M. *et al.* (2006) *C. elegans* BAF-1 and its kinase VRK-1 participate directly in postmitotic nuclear envelope assembly. *EMBO J.* 26, 132–143

31 Montes de Oca, R. *et al.* (2005) Binding of barrier to autointegration factor (BAF) to histone H3 and selected linker histones including H1.1. *J. Biol. Chem.* 280, 42252–42262
32 Dechat, T. *et al.* (2004) LAP2α transiently localizes to telomeres and to defined chromatin regions during nuclear assembly. *J. Cell Sci.* 117, 6117–6128
33 Segura-Totten, M. *et al.* (2002) Barrier-to-autointegration factor: major roles in chromatin decondensation and nuclear assembly. *J. Cell Biol.* 158, 475–485
34 Margalit, A. *et al.* (2005) A lamin-dependent pathway that regulates nuclear organization, cell cycle progression and germ cell development. *Novartis Found. Symp.* 264, 231–240
35 Mersfelder, E.L. and Parthun, M.R. (2006) The tale beyond the tail: histone core domain modifications and the regulation of chromatin structure. *Nucleic Acids Res.* 34, 2653–2662
36 Nili, E. *et al.* (2001) Nuclear membrane protein, LAP2b, mediates transcriptional repression alone and together with its binding partner GCL (germ cell-less). *J. Cell Sci.* 114, 3297–3307
37 Dorner, D. *et al.* (2006) Lamina-associated polypeptide 2α regulates cell cycle progression and differentiation via the retinoblastoma–E2F pathway. *J. Cell Biol.* 173, 83–93
38 Lin, F. *et al.* (2005) MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor-β signaling. *Hum. Mol. Genet.* 14, 437–445
39 Markiewicz, E. *et al.* (2006) The inner nuclear membrane protein emerin regulates β-catenin activity by restricting its accumulation in the nucleus. *EMBO J.* 25, 3275–3285
40 Holaska, J.M. *et al.* (2003) Transcriptional repressor germ cell-less (GCL) and barrier-to-autointegration factor (BAF) compete for binding to emerin *in vitro*. *J. Biol. Chem.* 278, 6969–6975
41 Tifft, K.E. *et al.* (2006) Barrier-to-autointegration factor-like (BAF-L): a proposed regulator of BAF. *Exp. Cell Res.* 312, 478–487
42 Bengtsson, L. and Wilson, K.L. (2006) BAF phosphorylation on Ser-4 regulates emerin binding to lamin A *in vitro* and emerin localization *in vivo*. *Mol. Biol. Cell* 17, 1154–1163
43 Hirano, Y. *et al.* (2005) Dissociation of emerin from barrier-to-autointegration factor is regulated through mitotic phosphorylation of emerin in a *Xenopus* egg cell-free system. *J. Biol. Chem.* 280, 39925–39933
44 Kimura, T. *et al.* (2003) Mouse germ cell-less as an essential component for nuclear integrity. *Mol. Cell. Biol.* 23, 1304–1315
45 Gruenbaum, Y. *et al.* (2005) The nuclear lamina comes of age. *Nat. Rev. Mol. Cell Biol.* 6, 21–31
46 Stuurman, N. *et al.* (1998) Nuclear lamins: their structure, assembly, and interactions. *J. Struct. Biol.* 122, 42–66
47 Lin, F. *et al.* (2000) MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J. Biol. Chem.* 275, 4840–4847
48 Broers, J.L. *et al.* (2006) Nuclear lamins: laminopathies and their role in premature ageing. *Physiol. Rev.* 86, 967–1008
49 Worman, H.J. and Courvalin, J.C. (2005) Nuclear envelope, nuclear lamina, and inherited disease. *Int. Rev. Cytol.* 246, 231–279
50 Gotzmann, J. and Foisner, R. (2005) A-type lamin complexes and regenerative potential: a step towards understanding laminopathic diseases? *Histochem. Cell Biol.* 125, 33–41
51 Taylor, M.R. *et al.* (2005) Thymopoietin (lamina-associated polypeptide 2) gene mutation associated with dilated cardiomyopathy. *Hum. Mutat.* 26, 566–574
52 Laguri, C. *et al.* (2001) Structural characterization of the LEM motif common to three human inner nuclear membrane proteins. *Structure* 9, 503–511
53 Brachner, A. *et al.* (2005) LEM2 is a novel MAN1-related inner nuclear membrane protein associated with A-type lamins. *J. Cell Sci.* 118, 5797–5810
54 Lee, K.K. and Wilson, K.L. (2004) All in the family: evidence for four new LEM-domain proteins Lem2 (NET-25), Lem3, Lem4 and Lem5 in the human genome. *Symp. Soc. Exp. Biol.* 56, 329–339
55 Padan, R. *et al.* (1990) Isolation and characterization of the *Drosophila* nuclear envelope otefin cDNA. *J. Biol. Chem.* 265, 7808–7813
56 Wagner, N. *et al.* (2006) The *Drosophila melanogaster* LEM-domain protein MAN1. *Eur. J. Cell Biol.* 85, 91–105
57 Lee, K.K. *et al.* (2000) *C. elegans* nuclear envelope proteins emerin, MAN1, lamin, and nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis. *Mol. Biol. Cell* 11, 3089–3099
58 Burke, B. and Stewart, C.L. (2002) Life at the edge: the nuclear envelope and human disease. *Nat. Rev. Mol. Cell Biol.* 3, 575–585
59 Foisner, R. (2001) Inner nuclear membrane proteins and the nuclear lamina. *J. Cell Sci.* 114, 3791–3792
60 Goldman, R.D. *et al.* (2002) Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* 16, 533–547
61 Berger, R. *et al.* (1996) The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. *Genome Res.* 6, 361–370
62 King, M.C. *et al.* (2006) Karyopherin-mediated import of integral inner nuclear membrane proteins. *Nature* 442, 1003–1007
63 Rodriguez-Navarro, S. *et al.* (2002) SRC1: an intron-containing yeast gene involved in sister chromatid segregation. *Yeast* 19, 43–54

2.7 Aims of the Thesis

Since the discovery that a heterogeneous group of human diseases termed "envelopathies" is genetically linked to the nuclear lamina and INM proteins, these proteins became a major focus in the field of nuclear research. Among about 60 different INM proteins, a subset of proteins contains a specific structural motif called LEM domain. Defects in three known LEM proteins, LAP2, Emerin and MAN1, were linked to human pathologies included in the group of envelopathies. Novel members of the LEM domain containing protein family are therefore considered to be potential candidates for disease causing genes involved in human pathologies. This notion is further supported by the fact that only a limited percentage of investigated cases of clinically diagnosed envelopathies revealed mutations in known proteins of the nuclear lamina or INM.

As a detailed knowledge on the expression, interactions and properties of these proteins is a prerequisite for understanding the molecular basis of these diseases, the aim of this thesis was the identification and functional characterization of novel mammalian LEM proteins.

3. Results

3.1. Evolution of the LEM Domain Containing Protein Family

The increasing availability of databases providing complete sequence data of many organisms allowed us to perform a screen for evolutionary conserved LEM domain proteins and to identify potentially important regions within these proteins by sequence alignments.

In general the number of different LEM domain proteins increased during evolution, reaching a predicted number of seven individual genes within the genome of mammalian species. These include Emerin, LAP2, MAN1, LEM2, LEM3, LEM4, and LEM5 (Table 2). LEM2 was the only gene identified to have an ortholog in yeast, whereas four genes (Emerin, LEM2, LEM3 and LEM4) are present in *C.elegans*. Although genomic databases still contain some sequencing gaps, evolutionary models about the origin and growth of the LEM-domain containing protein family can be deduced.

The first LEM domain containing protein that appeared in evolution might have been a LEM2 precursor, which probably originated in an ancestral unicellular organism. Recently two putative LEM2 orthologous proteins have been identified in *S.cerevisiae*, *SRC1* (Brachner *et al.* 2005; Rodriguez-Navarro *et al.* 2002) (also termed *HeH1*) and *HeH2* (King *et al.* 2006). *HeH1* was consequently found also in several other unicellular organisms (see also chapter 3.2.3 and Fig. 17). LEM2 and *SRC1* share a common domain topology with one major difference: The N-terminal LEM motif of LEM2 is substituted by a SAP-like motif in *SRC1* and *HeH2* at the corresponding position. Since there exists no orthologous BAF protein in unicellular organisms (which binds to the LEM motif), a co-evolution of the LEM domain and BAF in early metazoan species seems a conceivable model (Margalit *et al.* 2007a). The switch from a direct binding to DNA of the SAP motif to an indirect binding of the LEM motif to DNA via BAF during evolution implies the generation of additional levels of regulation in protein-chromatin interactions during evolution. So far proteins containing a canonical LEM domain were found in *C.elegans* as the “earliest” organism, including three different proteins: ce-Emerin, ce-LEM-2 (previously termed: ce-MAN1) and ce-LEM-3 (Lee *et al.* 2000). Interestingly, in LAP2 proteins a LEM-like domain was identified which has the capability to bind directly to DNA (Cai *et al.* 2001) and which is more closely related to the SAP motif than to the LEM domain according to their calculated phylogenetic distance (Fig. 16). Although the LAP2 gene is only present in higher eukaryotes today (Table 2), the LEM-like motif might represent an intermediate structure which evolved at the transition from a SAP-like motif to the BAF-binding LEM motif and was preserved for unknown reasons in LAP2 proteins (no other proteins harboring a LEM-like motif have been found so far).

	Emerin	*LEM2*	*LEM3 / ANKRD41*	*MAN1 / LEMD3*	*LEM4**	*LAP2*	*LEM5 / LEMD1*	*BAF, BAF-like*	*A-type Lamins*	*B-type Lamins*
S.cerevisiae	-	SRC1 HeH2	-	-	-	-	-	-	-	-
C.elegans	emerin	lem-2	lem-3	-	Y55F3BR **	-	-	baf	-	lamin
D.melanogaster	- ***	-	LEM3*	MAN1	SD02148p **	-	-	BAF	LamC	LamDm$_0$
C.intestinalis	nd	nd	LEM3*	MAN1*	LEM4*	nd	nd	BAF	nd	Lamin 1 Lamin 2
D.rerio	nd	nd	LEM3*	MAN1	LEM4*	LAP2	nd	BAF	nd	Lamin B1 Lamin B2
X.tropicalis	nd	nd	LEM3*	XMAN1 SANE	LEM4*	LAP2	nd	BAF	Lamin A Lamin III	Lamin B1 Lamin B2
O. anatinus	Emerin	nd	LEM3*	nd	LEM4*	LAP2	nd	BAF	nd	nd
M.domestica	nd	LEM2*	LEM3*	nd	LEM4*	LAP2	nd	BAF	nd	nd
D. novemcinctus	nd	nd	LEM3*	MAN1*	LEM4*	LAP2	LEMD1	BAF	nd	nd
E.telfairi	nd	nd	LEM3*	MAN1*	LEM4*	LAP2	nd	BAF	nd	nd
R.norvegicus	Emerin	LEM2	LEM3	MAN1	LEM4	LAP2	LEMD1	BAF BAF-L	Lamin A/C	Lamin B1 Lamin B2
M.musculus	Emerin	LEM2	LEM3	MAN1	LEM4	LAP2	LEMD1	BAF BAF-L	Lamin A/C	Lamin B1 Lamin B2
H.sapiens	Emerin	LEM2	LEM3	MAN1	LEM4	LAP2 α,β,γ,δ,ε,ζ	LEMD1 a,b,c,d,e	BAF BAF-L	Lamin A/C	Lamin B1 Lamin B2

Table 2: LEM proteins present in selected organisms according to genomic database analyses. (*) Sequence present, but not annotated, (**) Sequence identified to be related to mammalian LEM4, but lacks predicted LEM-domain, (***) Two Arthropoda-specific proteins, Otefin and Bocksbeutel, are similar to the Emerin proteins. (nd) not determined due to sequencing gaps in genomic databases.

The alignment and calculation of the evolutionary relationship of all available sequences containing a LEM domain, revealed two related subgroups (Fig. 17) and three rather unrelated genes.

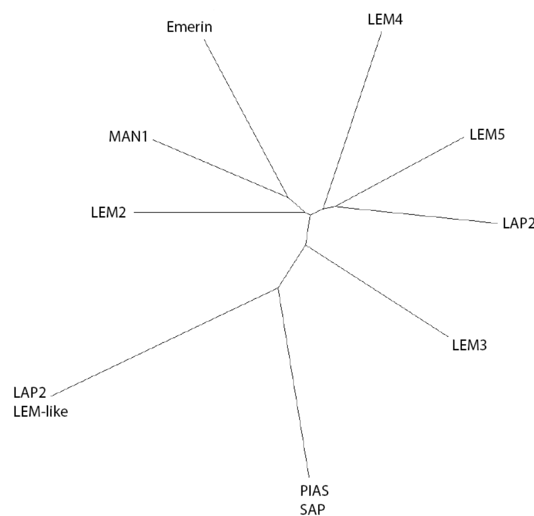


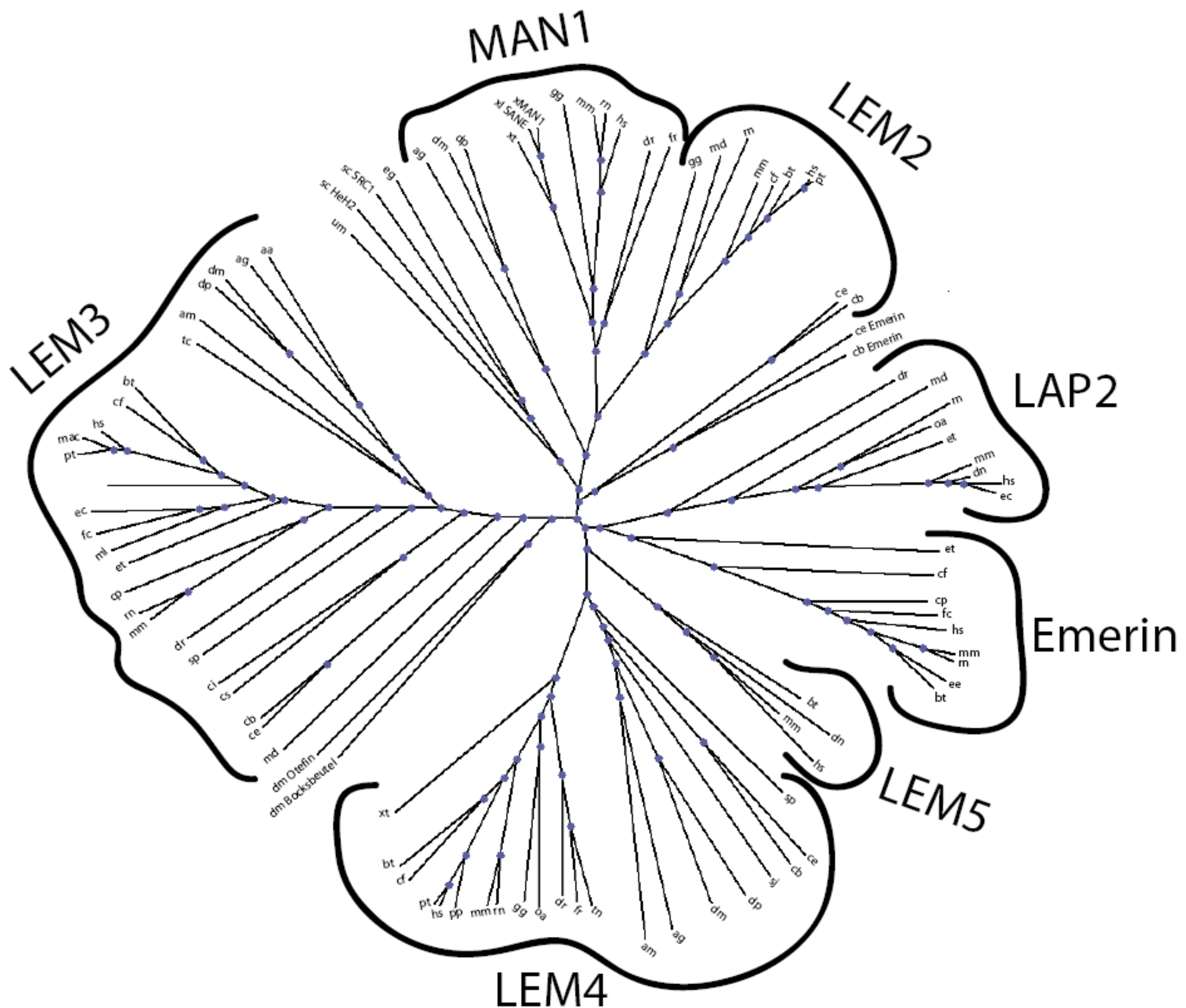
Figure 16: Phylogenetic relation of human LEM, LEM-like and SAP motifs. Unrooted tree diagram calculated from sequence homologies between the LEM, LEM-like and SAP motifs of the indicated proteins.

(1) LEM2 and MAN1 are highly related proteins and comprise a subgroup within the LEM protein family, which may have evolved from an early INM protein, which is still present in current unicellular eukaryotes. During evolution of metazoan organisms the MAN1 sequence emerged probably by a gene duplication of LEM2 and subsequently gained the MAN1-specific RRM motif, thereby adding a non-redundant unique function to MAN1. Interestingly the LEM2 sequence could not be found in the genomic datasets of *Arthropoda* (including insects), *Amphibia* (including frogs) and *Pisces* (including fishes), which could be explained by a loss of LEM2 during the evolution of these phyla or the LEM2 encoding genomic regions are not completely covered and sequenced yet. So far, *Gallus gallus* (chicken) was the "earliest" species identified containing both, LEM2 and MAN1 (Table 2 and Figure 15).

(2) Emerin and LAP2 seem to have a common origin as well, of which Emerin was emerging earlier in evolution. Analogous to LEM2 and MAN1, LAP2 may have emerged by a gene duplication of Emerin.

LEM3, LEM4 and LEM5 (Lee and Wilson 2004) evolved more independently. In the case of LEM4, the LEM domain evolved later in evolution, giving rise to two types of LEM4-related proteins, one with and one without a LEM motif. Based on primary sequence conservation, there are homologous genes to human LEM4 in *C.elegans* and *D.melanogaster*, which lack the characteristic LEM domain and thus are not considered members of the family of LEM domain

proteins (Table 2 and Fig. 17). Nevertheless, particular functions independent of the presence of a LEM motif might be conserved among all LEM4 related proteins. Therefore, data about these LEM4-related proteins might help elucidating the functions of LEM4 in higher eukaryotes.



aa	Aedes agypti (yellow fewer mosquito)
ag	Anopheles gambiae (malaria mosquito)
am	Apis melifera (honey bee)
bt	Bos taurus (cow)
ce	Caenorhabditis elegans
cb	Caenorhabditis briggsae
cf	Canis familiaris (dog)
ci	Ciona intestinalis (sea squirt)
cs	Ciona savyngi
dn	Dasypus novemcinctus (nine-banded armadillo)
dm	Drosophila melanogaster (fruitfly)
dr	Danio rerio (zebrafish)
ee	Erinaceus europeus (european hedgehog)
et	Echinops telfairi (hedgehog tenrec)
fr	Fugu rubripes (pufferfish)
gg	Gallus gallus (chicken)
hs	Homo sapiens (man)
la	Loxodonta africanus (african elephant)
mac	Macaca mulatta (rhesus monkey)
md	Monodelphis domestica (short-tailed opossum)
ml	Myotis lucifugus (brown bat)
mm	Mus musculus (mouse)
pp	Pongo pygmaeus
pt	Pan troglodytes (chimpanzee)
rn	Rattus norvegicus (brown rat)
sc	Saccharomyces cerevisiae (baker yeast)
sp	Strongylocentrotus purpuratus (purple sea urchin)
tn	Tetraodon nigroviridis
xl	Xenopus laevis (african clawed frog)
xt	Xenopus tropicalis

Figure 17: Evolution of the LEM-domain containing protein family. Unrooted tree diagram calculated from protein sequence alignments.

Except for LAP2, MAN1 and LEM5 all LEM proteins are present in *C.elegans*, indicating that this conserved family of proteins fulfils basic functions in multicellular organisms and may additionally confer functional redundancy to protect against genetic alteration induced loss-of-function. A comparison of the predicted domain topology of all members of the LEM protein family reveals that, with only one exception, the predicted LEM domain is located either at the very N-terminus (Emerin, MAN1, LEM2 and LEM5) or within the N-terminal part (LAP2, LEM4) of the proteins. The same holds true for the LEM-like motif in LAP2 proteins. LEM3 represents the only family member with a LEM domain located close to the middle of the protein sequence (Fig. 18).

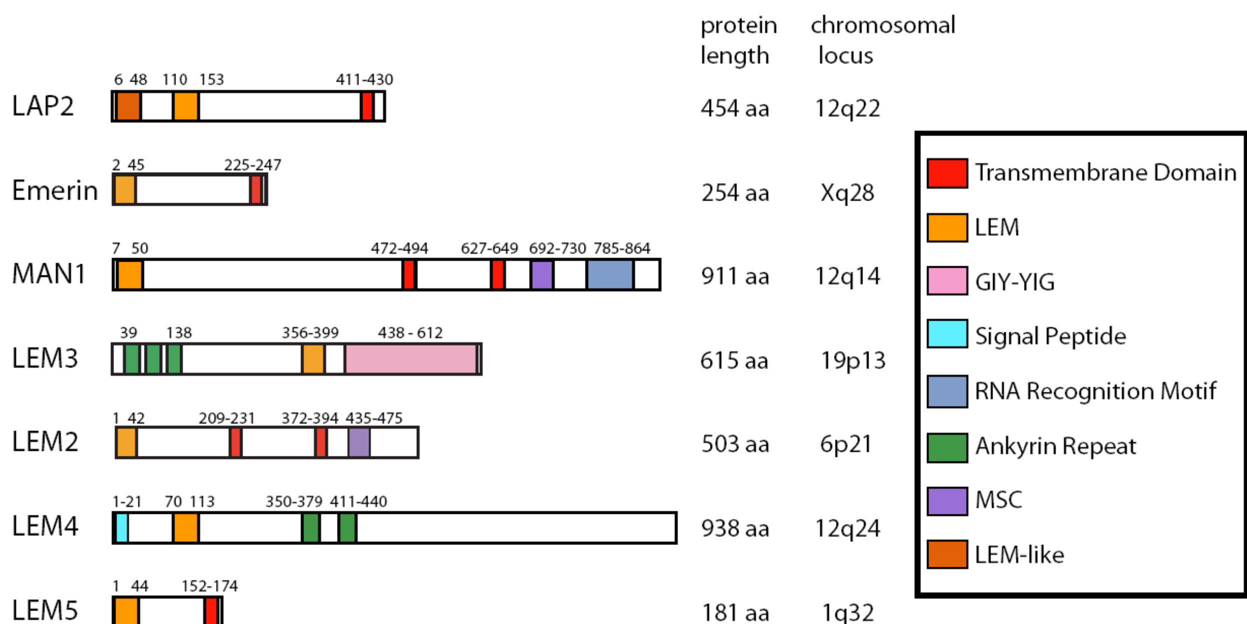


Figure 18: Predicted domain topology of the human LEM-domain containing protein family. Schematic protein topology (to scale) according to computational predictions, numbers indicate positions within the protein sequence. Chromosomal loci are indicated according to the ENSEMBL human genome database (http://www.ensembl.org).

Whether the preferential localization of the LEM domain at the N-terminus of proteins is due to structural or functional reasons or both is unknown but it might be due to the fact that most of these proteins are anchored within the INM via one or two transmembrane domains located within the middle or C-terminal part (Fig. 18 and Table 3).

For proteins with a single transmembrane domain, the N-terminal tail resides in the nucleoplasm thus all interactions with nuclear lamins, BAF or other nuclear proteins are restricted to the nucleoplasmic N-terminal part. In case of LEM2 and MAN1, which contain two transmembrane regions, both N-and C-terminus reside in the nucleoplasm and have the possibility to interact with nuclear components. The LEM motif is located at the N-terminus in both proteins and

additional functional motifs were predicted within the C-terminal part of the proteins (Fig. 18, and chapters 3.2.1 and 3.2.2).

	Subcellular Localization	Reference
LAP2	INM, except LAP2α which localizes in the nucleoplasm	(Dechat *et al.* 1998; Furukawa *et al.* 1995)
Emerin	INM	(Manilal *et al.* 1996)
MAN1	INM	(Lin *et al.* 2000)
LEM2	INM	(Brachner *et al.* 2005)
LEM3	nucleo-cytoplasmic shuttling protein	unpublished, see chapter 3.3.1
LEM4	unknown; *in silico* prediction: nuclear	unpublished
LEM5	INM	(Yuki *et al.* 2004)

Table 3: Subcellular localization of human LEM proteins as far as known from literature or in silico prediction.

3.2 LEM2

LEM2 was identified in database screens as a putative novel LEM domain containing protein in mammalian organisms and was retrieved independently in a proteomic screen for novel, INM proteins (Schirmer *et al.* 2003). We cloned and analyzed human and murine LEM2 in order to verify its predicted characteristics and to detect potential laminopathy-related features. Initial localization studies using immunofluorescence and protein extraction experiments approved that this novel LEM protein is indeed a resident protein of the INM. All LEM domain containing proteins locating at the INM or in the nucleoplasm have been shown to interact with the nuclear lamins. Therefore, it was probable that also LEM2 interacts with lamins. We determined the lamin binding properties of LEM2 via *in vivo* and *in vitro* approaches and could show that LEM2 N-terminus is interacting with the Lamin A/C tail region in a direct manner.

Further on we observed two phenomena in cells overexpressing human LEM2. First, LEM2 accumulates in patch-like structures at the NE and recruits Lamin A/C, BAF, Emerin as well as MAN1 to these patches, which likely represent large invaginations of the NE, as determined by transmission electron microscopy. Secondly, LEM2 affects nuclear envelope reassembly after mitosis as we frequently observed daughter nuclei still connected by a membranous bridge, although cells have completed cytokinesis (see chapter 3.2.1).

Analysis of LEM2 fragments revealed a dominant inhibitory effect on cell cycle of LEM2 N-terminus, which could be provoked by its interaction with nucleoplasmic Lamin A/C thereby perturbing the complex of Lamin A, LAP2α and the tumor suppressor pRB, which results in a cell cycle arrest. In contrast, overexpression of full length LEM2 was even accelerating cell proliferation indicating that localization and interactions with Lamin A/C and BAF in the nuclear interior or at the periphery causes entirely different effects (see chapter 3.2.2).

Additionally we investigated the phylogenetic relationship of LEM2 and its proposed orthologous protein in yeast, *SRC1*, on a functional level. Therefore, we collaborated with the lab of Susana Rodriguez-Navarro, who performed complementation assays with human LEM2 in *SRC1*$^{-/-}$ yeast strains and could show that hLEM2 indeed is able to partially rescue the phenotype of the knockout strain. Additionally we performed localization studies of fluorescently tagged human LEM2 in yeast and found LEM2 to be targeted to the yeast NE. Taken together these results provide strong evidence for evolutionary conserved functions within the LEM2/SRC1 superfamily (see chapter 3.2.3).

3.2.1 LEM2 is a Novel MAN1-related Inner Nuclear Membrane Protein Associated with A-type Lamins

LEM2 is a novel MAN1-related inner nuclear membrane protein associated with A-type lamins

Andreas Brachner[1], Siegfried Reipert[2], Roland Foisner[1,*] and Josef Gotzmann[1,*]

[1]Department of Medical Biochemistry, Max F. Perutz Laboratories, Vienna Biocenter, Medical University of Vienna, Dr Bohrgasse 9/3, A-1030 Vienna, Austria

[2]Department of Molecular Cell Biology, University of Vienna, Max F. Perutz Laboratories, Vienna Biocenter, Dr Bohrgasse 9/3, A-1030 Vienna, Austria

*Authors for correspondence (e-mail: roland.foisner@meduniwien.ac.at; josef.gotzmann@meduniwien.ac.at)

Accepted 20 September 2005
Journal of Cell Science 118, 5797-5810 Published by The Company of Biologists 2005
doi:10.1242/jcs.02701

Journal of Cell Science

Summary

The LEM (lamina-associated polypeptide–emerin–MAN1) domain is a motif shared by a group of lamin-interacting proteins in the inner nuclear membrane (INM) and in the nucleoplasm. The LEM domain mediates binding to a DNA-crosslinking protein, barrier-to-autointegration factor (BAF). We describe a novel, ubiquitously expressed LEM domain protein, LEM2, which is structurally related to MAN1. LEM2 contains an N-terminal LEM motif, two predicted transmembrane domains and a MAN1-Src1p C-terminal (MSC) domain highly homologous to MAN1, but lacks the MAN1-specific C-terminal RNA-recognition motif. Immunofluorescence microscopy of digitonin-treated cells and subcellular fractionation identified LEM2 as a lamina-associated protein residing in the INM. LEM2 binds to the lamin C tail in vitro. Targeting of LEM2 to the nuclear envelope requires A-type lamins and is mediated by the N-terminal and transmembrane domains. Highly overexpressed LEM2 accumulates in patches at the nuclear envelope and forms membrane bridges between nuclei of adjacent cells. LEM2 structures recruit A-type lamins, emerin, MAN1 and BAF, whereas lamin B and lamin B receptor are excluded. Our data identify LEM2 as a novel A-type-lamin-associated INM protein involved in nuclear structure organization.

Key words: Chromatin organization, Lamina-associated proteins, Lamins, LEM domain, Nuclear architecture, Nuclear envelope

Introduction

The nuclear envelope (NE) separates nuclear and cytoplasmic compartments. It is composed of an inner (INM) and an outer (ONM) nuclear membrane that are separated by a luminal space and are periodically connected at nuclear pore complexes, as well as a filamentous meshwork near the INM termed the nuclear lamina (Foisner, 2003; Gruenbaum et al., 2005). The nuclear lamina is found in multicellular animals. It is formed by type V intermediate filaments including B-type lamins, which are expressed in all cells and are essential for viability (Vergnes et al., 2004), and A-type lamins, the expression of which is limited to differentiated cells (Goldman et al., 2002). Several integral proteins of the INM bind to and are tightly integrated in the lamina scaffold (Burke and Stewart, 2002; Foisner, 2001; Gruenbaum et al., 2005). In particular, the lamin B receptor (LBR) and LAP2β specifically bind to B-type lamins (Foisner and Gerace, 1993; Furukawa et al., 1998; Worman et al., 1988; Ye and Worman, 1994), whereas LAP1, emerin and nesprins have been shown to associate primarily with A-type lamins (reviewed by Zastrow et al., 2004).

A group of the lamin-binding proteins share a common structural motif of 40 amino acids (aa) at their N-termini, called the lamina-associated polypeptide–emerin–MAN1 (LEM) domain (Cai et al., 2001; Laguri et al., 2001; Lin et al., 2000), which mediates the interaction with a highly conserved DNA-crosslinking protein, barrier-to-autointegration factor (BAF) (Cai et al., 2001; Furukawa, 1999; Lee et al., 2001; Mansharamani and Wilson, 2005; Segura-Totten and Wilson, 2004; Shumaker et al., 2001). The founding members of the LEM-domain protein family include several alternatively spliced LAP2 isoforms (Berger et al., 1996; Dechat et al., 2000b; Furukawa et al., 1995; Harris et al., 1994), emerin, and MAN1 (Lin et al., 2000). The LAP2 isoforms contain an additional LEM-like motif at the extreme N-terminus, which interacts with DNA (Cai et al., 2001). Other members of the LEM-domain protein family include the *Drosophila*-specific proteins otefin (Ashery-Padan et al., 1997) and Bocksbeutel (Wagner et al., 2004), and the uncharacterized *Caenorhabditis elegans* LEM3 (Lee et al., 2000). A comprehensive database screen identified a mammalian orthologue of Ce-LEM3 and three novel LEM-domain proteins in higher eukaryotes, which were termed LEM2, LEM4 and LEM5 based on and extending the *C. elegans* nomenclature (Lee and Wilson, 2004; Mansharamani and Wilson, 2005).

On the basis of their interaction with BAF and DNA, the LEM-domain proteins have been implicated in chromatin organization (Dechat et al., 2004; Haraguchi et al., 2001; Liu et al., 2003; Segura-Totten et al., 2002; Segura-Totten and Wilson, 2004; Shimi et al., 2004). Emerin and LEM2 might have overlapping and redundant functions, as RNA interference-mediated knockdown of both proteins in *C. elegans* was embryonic lethal at the 100-cell stage, whereas single knockouts showed no or less-severe phenotypes (Liu et al., 2003). Furthermore, emerin and MAN1 were found to bind

to each other in vitro (Mansharamani and Wilson, 2005). Numerous novel recently identified binding partners of LEM-domain proteins indicate many important functions of these proteins in diverse cellular processes (Bengtsson and Wilson, 2004; Gruenbaum et al., 2005). LAP2β and emerin bind the transcriptional repressor germ-cell less (Holaska et al., 2003; Nili et al., 2001), and LAP2α interacts with the tumor suppressor retinoblastoma protein (Markiewicz et al., 2002), implicating functions in transcriptional regulation. Other potential functions of LEM-domain proteins can be envisaged based on the observation that emerin can act as a capping protein for nuclear actin (Holaska et al., 2004). Interestingly, XMAN1, the *Xenopus* orthologue of human MAN1, has been shown to serve as antagonist of the bone morphogenetic protein (BMP) signalling pathway by binding to downstream regulatory Smads 1, 5 and 8 (Osada et al., 2003). Similarly, human MAN1 can augment BMP signalling as well as signalling through transforming growth factor β (TGF-β) and activin by binding to Smads 1, 2 and 3, but not Smad 4 (Hellemans et al., 2004; Lin et al., 2005; Pan et al., 2005).

Mutations in genes encoding A-type lamins and some of their binding partners give rise to a diverse group of diseases termed 'laminopathies' (Burke and Stewart, 2002; Gotzmann, 2004; Gruenbaum et al., 2005; Hutchison and Worman, 2004; Mounkes and Stewart, 2004). The disease phenotypes are diverse, affecting skeletal muscles, heart, adipose, bone, skin and neuronal tissues, and also include premature aging; the underlying molecular mechanisms for the laminopathies are still unclear. Apart from disease-causing mutations in the *LMNA* gene, mutations in genes encoding the LEM-domain proteins emerin (Bione et al., 1994; Emery, 1987; Emery and Dreifuss, 1966; Manilal et al., 1996) and MAN1 (Hellemans et al., 2004) give rise to muscular dystrophy and bone-related diseases, respectively. Since some disease-linked mutations in *LMNA* or lack of lamin A cause mislocalization of emerin to the endoplasmic reticulum (ER) (Holt et al., 2003; Muchir et al., 2004; Ostlund et al., 2001; Raharjo et al., 2001; Sullivan et al., 1999; Vaughan et al., 2001), it is likely that disruption of lamin A complexes caused by mutations in either lamin A/C or in associated proteins are the molecular cause of these diseases. Therefore, in order to gain more insights into the functions of lamin A/C complexes and their involvement in disease, it is extremely important to identify and characterize novel proteins of these complexes.

Here we describe and characterize a novel human MAN1-related LEM-domain protein, LEM2. Importantly, we demonstrate that LEM2 binds to lamin C and requires association with A-type lamins for proper NE localization. Furthermore, highly overexpressed LEM2 formed clusters and membranous invaginations at the NE as well as tubular structures interlinking nuclei of adjacent cells, to which it recruits lamin A/C and associated proteins emerin and BAF. This suggests a role of LEM2 in the organization of lamin A/C complexes.

Materials and Methods

Cell culture and transfection

The cell lines HeLa, COS, MDCK and C2C12, as well as mouse embryonic fibroblasts (MEFs) from wild-type or *Lmna*$^{-/-}$ mice [kindly supplied by C. Stewart, NCI-Frederick, USA (Sullivan et al., 1999)] were routinely cultivated in DMEM supplemented with 10% fetal calf serum (Invitrogen), penicillin/streptomycin and 2 mM L-Glutamine at 37°C in a humidified atmosphere containing 5% CO_2. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa- (HeLa-LEM2), COS7- (COS-LEM2) and C2C12- (C2C12-LEM2) cells stably expressing hLEM2 were cloned by selecting for resistance to the antibiotic blasticidin (30 μg/ml; Invitrogen).

Antibodies

Mouse and rabbit anti-V5 antibodies were purchased from Invitrogen and Sigma, respectively. Rabbit anti-phospho-histone-H3 antibody was obtained from Upstate Biotechnologies; mouse anti-α-tubulin antibody and a polyclonal serum against actin were from Sigma; mouse monoclonal anti-lamin A (clone#133A2) was from Abcam; and mouse anti-NUP62 was from BD Pharmingen. The rabbit antiserum to LAP2α has been described previously (Vlcek et al., 2002). The antiserum to BAF was a generous gift of K. Furukawa [Niigata University, Japan (Furukawa, 1999)]. The antiserum to ER-marker α-calnexin was provided by E. Ivessa [Medical University Vienna, Austria (de Virgilio et al., 1998)]. The guinea pig anti-LBR antibody was provided by H. Hermann [DKFZ, Heidelberg, Germany (Dreger et al., 2002)]. The mouse anti-lamin B1 monoclonal antibody 8D1 was provided by D. Vaux [Sir William Dunn School of Pathology, Oxford, UK (Maske and Vaux, 2004)].

Plasmids and cloning strategy

A cDNA clone (#T5554) containing full-length human LEM2 was obtained from Genecopoeia (Germantown, USA) and used as template to amplify cDNA of full-length human LEM2 or the N-terminus (LEM2-NT) using the following primers: full-length, 5′-CACCATGGCCGGCCTGTCGGACCTGGAACTGCGGC-3′ and 5′-GCATCGCTCTGAGTCAGAGAAGGAAGAGG-3′; LEM2-NT, 5′-CACCATGGCCGGCCTGTCGGACCTGGAACTGCGGC-3′ and 5′-AAGCTTCGCGCCAGCAGGGCCCGCTCGAGT-3′. cDNAs were cloned into pENTR/D-TOPO (Invitrogen). For eukaryotic expression, hLEM2 and LEM2-NT were shuttled using the LR-recombination reaction (Invitrogen) into pTRACER-B, made Gateway®-compatible by insertion of the 'conversion cassette' (Invitrogen) into the *Eco*RV site. Full-length human MAN1 was amplified by PCR (5′-CACCA-TGGCGGCGGCAGCAGCTTC-3′; 5′-GCAGGAACTTCCTTGAG-AAT-3′) and cloned into pTRACER-B as above. Vectors expressing deletion mutants of hLEM2 were constructed by restriction digest and re-ligation using pTRACER with full-length hLEM2. For LEM2ΔCT (Δaa 378-486) we used *Msl*I, for LEM2ΔNT (Δaa 130-203) *Sfo*I, for LEM2-LEM (Δaa 74-503) *Bss*HII, and for LEM2ΔLEM+NT (Δaa 28-203) *Sma*I and *Sfo*I. All expression constructs were tagged with a C-terminal V5 epitope. For the LEM2-GFP construct, a *Spe*I-*Xba*I fragment from the pTRACER vector expressing full-length LEM2 was subcloned into the *Nhe*I site of peGFP-C1 (Clontech). A construct expressing GFP-tagged pre-lamin A (Sullivan et al., 1999) was a generous gift of C. Stewart; the plasmid GFP-xLaminB1Δ2+ was kindly provided by C. Hutchison [University of Durham, UK (Izumi et al., 2000)].

Subcellular fractionation, gel electrophoresis and immunoblotting

Subcellular fractionation was done essentially as described (Dechat et al., 1998; Gotzmann et al., 2000). In brief, cells were broken in hypotonic buffer using a dounce homogenizer with a tight-fitting pestle. After addition of 8% sucrose, the soluble cytoplasmic and insoluble nuclear fractions were separated by centrifugation at 2000 *g* for 15 minutes at 4°C. The nuclei-containing pellet fractions were extracted in the same buffer supplemented with 1% Triton X-100, or

200 mM NaCl, or combinations of both, or 7 M urea followed by centrifugation at 15,000 *g* for 10 minutes; analyses of supernatant and pellet fractions were carried out by western blotting. SDS-PAGE and immunoblotting was performed essentially as described previously (Gotzmann et al., 2002; Gotzmann et al., 2000).

PCR analysis

Total RNA was purified by standard techniques from whole mouse embryos (dpc 12, 14, 16, 18) or purchased as total RNA collections of human and mouse tissues (Clontech). Poly(A)$^+$ mRNA was extracted from total RNA with an mRNA Isolation Kit and reverse transcribed using the first-strand cDNA Synthesis Kit (both from Roche). Aliquots of the resulting products were employed as templates for specific PCR amplifications using Ready-To-Go PCR beads (Amersham Pharmacia Biotech). The conditions for the PCR reactions were optimized for each primer pair. All cDNAs were normalized according to actin expression levels. Real-time PCR analysis of LEM2 expression at developmental stages was performed using an iCycler real-time PCR detection system (Bio-Rad) and the DNA-binding dye SYBR Green I. The following pairs of forward and reverse primers (all in 5′-3′ direction) were used for amplifications: human and mouse actin, ATCTGGCACCACACCTTCTAC and CAGCCAGGTCCAGACGCAGG; human LEM2, GCCGGCCTG-TCGGACCTGGAACT and GGCGGCGCAGCTTGTTGCGGTAG; mouse LEM2, AAGCGAGTATGGGACCGTGCTGTG and AGGT-GAGACCCGGCTGAAGAGTTG.

Blot overlay assay

Full-length hLEM2 and an N-terminal fragment (LEM2-NT; aa 46-195) of hLEM2 were subcloned into a pET102 vector backbone using TOPO technology (Invitrogen). Expression plasmids for lamin C fragments (head, aa 1-171; rod, aa 171-319; tail, aa 319-572) and full-length LAP2α are described elsewhere (Dechat et al., 2000a). Coupled transcription and translation in the presence of [^{35}S]-methionine was performed using the TNT® Quick Coupled Transcription/Translation according to the manufacturer's instructions (Promega). Recombinant lamin C fragments and LAP2α were separated by SDS-PAGE and transferred onto nitrocellulose. Proteins were stained with PonceauS, and blots were incubated in overlay buffer (10 mM Hepes, pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 0.1% Triton X-100, 1 mM DTT) for 3 hours, blocked with 5% skim milk in overlay buffer, and probed with whole reticulocyte lysate containing ^{35}S-labelled proteins diluted 1:50 in overlay buffer with 1% skim milk and 1 mM phenylmethylsulphonylfluoride (PMSF) overnight at 4°C. After extensive washing in phosphate-buffered saline/Tween (PBST), bound proteins were detected by autoradiography.

Immunofluorescence microscopy

Cells were grown on poly-L-lysine-coated glass coverslips, fixed in methanol at –20°C for 3 minutes, or fixed in 4% paraformaldehyde for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Digitonin extraction of fixed cells was done with 40 μg/ml digitonin (Calbiochem) in PBS for 3 minutes on ice. Cells were blocked in 0.5% gelatin in PBS for 15 minutes, incubated with primary antibodies for 45 minutes, washed and probed with the appropriate secondary antibodies conjugated to either Texas Red (Jackson Immuno Research) or Alexa Fluor 488 (Molecular Probes) for 45 minutes. DNA was stained with Hoechst for 5 minutes, and samples were mounted in Mowiol (Fluka). Confocal images of the samples were taken with a confocal laser scanning microscope (LSM510 and Axiovert 100; Zeiss). Digital images were analysed and adjusted for brightness and contrast using the software LSM-Image-Browser (Zeiss) and Adobe Photoshop.

Electron microscopy

Cells grown on glass coverslips were fixed in 3% glutaraldehyde in 0.15 M Sorensen's buffer, pH 7.4, for 1 hour, incubated in 1% OsO_4 in Sorensen's for 1 hour and dehydrated with increasing concentrations of ethanol. Samples were subsequently 'flat' embedded in epoxy resin (Agar 100). Thin sections of 60-80 nm were cut with a LEICA Ultracut S ultramicrotome, mounted on copper grids, contrasted with uranyl acetate and lead citrate, and examined in a JEOL JEM-1210 electron microscope at 60 kV. Images were acquired using a Mega View III digital camera and analySIS FIVE software (SIS, Praha, Czech Republic).

Computer-assisted analysis

Alignments of cDNA sequences, genomic contig sequence alignments and database searches were performed by NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/) and ClustalW (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994). Genomic analysis was done using the ENSEMBL Genome Browser (http://www.ensembl.org/; Sanger Institute). The sequence of the murine orthologue of hLEM2 was calculated using the GENSCAN gene-prediction software [http://genes.mit.edu/GENSCAN.html (Burge and Karlin, 1997)]. Protein motifs and pattern searches were performed using SMART [http://smart.embl-heidelberg.de/ (Letunic et al., 2004; Schultz et al., 1998)], CDD (http://www.ncbi.nlm.nih.gov/; NCBI) and PSORT II (http://psort.nibb.ac.jp/form2.html). Transmembrane domains were calculated using the membrane protein topology database [http://blanco.biomol.uci.edu/mptopo/ (Jayasinghe et al., 2001)], the TMHMM 2.0 prediction software [http://www.cbs.dtu.dk/services/TMHMM/ (Krogh et al., 2001)], the SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/) and the DAS-TM filter algorithm (Cserzo et al., 2004). Phylogenetic tree predictions were done using eSHADOW [http://eshadow.dcode.org/ (Ovcharenko et al., 2004)] and visualized using the Phylodendron software (http://iubio.bio.indiana.edu/treeapp/).

Results

LEM2 is a novel mammalian LEM-domain protein structurally related to MAN1

Database searches for novel mammalian LEM-domain proteins revealed human (Acc. No.: NP_851853) and mouse (Acc. No.: AAT71319) LEM2, which show 83% and 87% overall aa sequence identity and similarity, respectively (Fig. 1A). The LEM domain in LEM2 was located at the very N-terminus of the molecule (aa 1-42; Fig. 1A, red box) and showed 97% sequence identity between human and mouse. The LEM domain in human LEM2 (hLEM2) is more closely related to the LEM domains described originally in hMAN1, hLAP2 and hEmerin (Lin et al., 2000) than to the LEM-like motif present in LAP2 (Fig. 1C). However, five residues within the LEM and LEM-like domains in these proteins were strictly conserved (Fig. 1C, red), two of which (P and Y) have been shown to be essential for the interaction with BAF in LAP2 (Shumaker et al., 2001). The hLEM2 polypeptide encompasses 503 aa (predicted molecular mass 56 kDa) and is highly basic (pI=9.2) mostly owing to a highly basic N-terminus (aa 1-209, pI=11.3). hLEM2 contains two predicted transmembrane domains between aa 209-231 and aa 372-394 (Fig. 1A, blue) and a MAN1-Src1p C-terminal (MSC) domain (Mans et al., 2004) between aa 435-493 (green). The gene encoding hLEM2 was annotated to chromosome 6p21.31, including 9 exons and 8 introns spanning about 18 kb (data not shown). The *Lem2* gene in

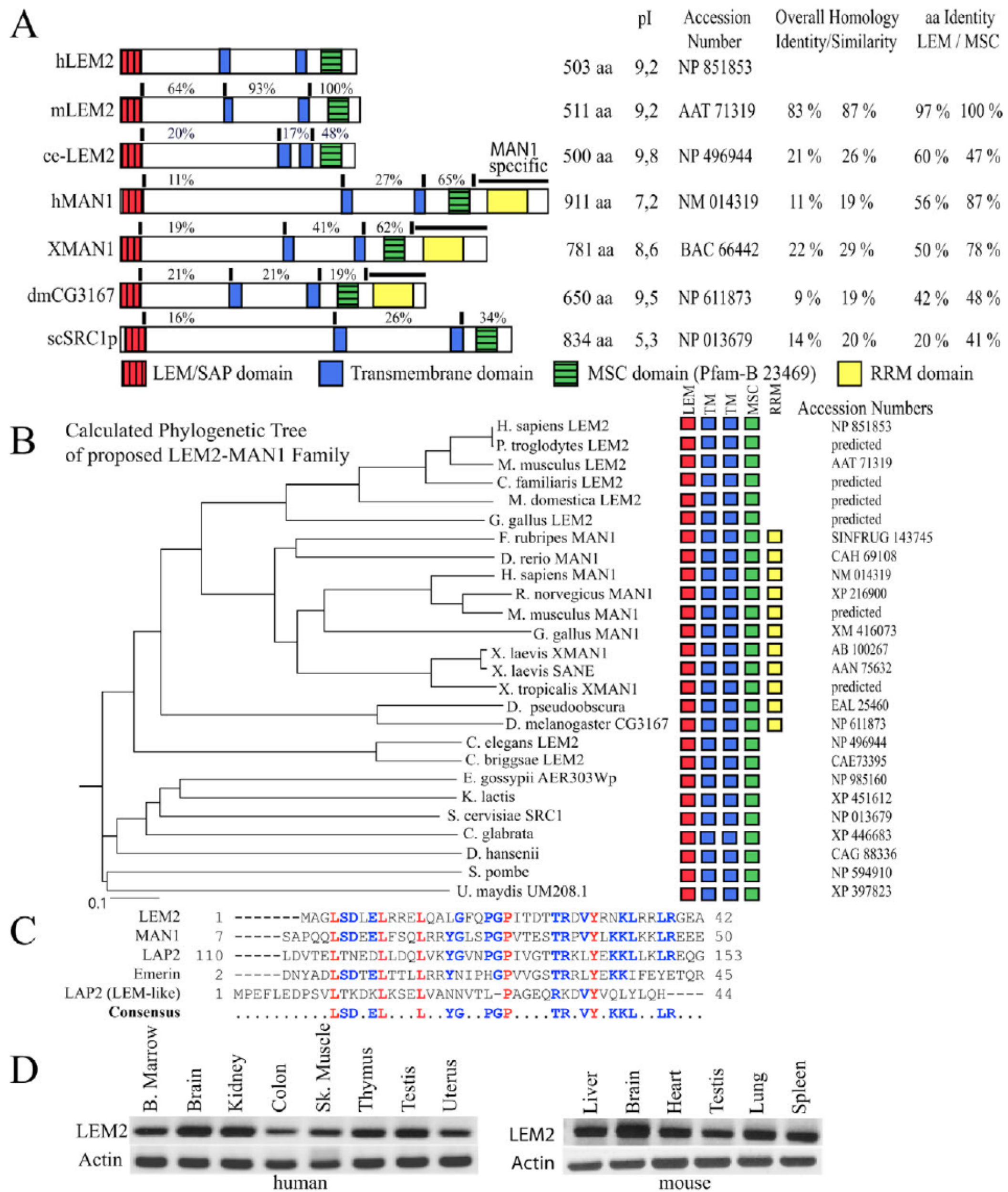


Fig. 1. LEM2 is a novel ubiquitously expressed member of the MAN1 subfamily within the LEM-domain proteins. (A) Comparison of the domain organization of human LEM2 (hLEM2) with orthologues of mouse (mLEM2) and *C. elegans* (Ce-LEM2), as well as with MAN1 proteins from human (hMAN1), *Xenopus laevis* (XMAN1), *Drosophila* (dmCG3167) and *Saccharomyces cerevisiae* (scSRC1p). Percentage identity of aa residues relative to human LEM2 are indicated within defined domains (intersected by small black bars), including the N-terminus, the lumenal part and the C-terminus. The MAN1-specific region is additionally marked by a black line. LEM/SAP domains are shown in red, transmembrane domains in blue, the MSC domain in green and the MAN1-specific RRM domain in yellow. (B) Cladogram displaying predicted phylogenetic divergence between members of a proposed LEM2-MAN1 family. The domain organization of the proteins is schematically indicated by the coloured boxes, as in (A). (C) Multiple sequence alignment of the LEM domains of hLEM2, MAN1, LAP2β, emerin and of the LEM-like motif in LAP2β. Invariant residues are in red, highly conserved in blue. Numbers indicate respective aa positions in the full-length proteins. (D) LEM2 mRNA levels in human and mouse tissues determined by semiquantitative RT-PCR. Actin mRNA levels were used for normalization. Agarose gels of PCR fragments are shown; B. Marrow, bone marrow; Sk. Muscle, skeletal muscle.

mouse was allocated to synthenic regions of chromosome 17A3.3.

We detected LEM2 orthologues in *C. elegans* (Fig. 1A), rat, chicken and dog (data not shown). The overall domain organization of hLEM2 is identical to that of hMAN1 and other MAN1 orthologues (e.g. *Xenopus* XMAN1), except that LEM2 lacked an RNA-recognition motif found in the C-termini of MAN1-type proteins (Fig. 1, RRM domain, yellow). Except for the LEM domain, hLEM2 and hMAN1 are less well conserved in their N-termini (upstream of the first predicted transmembrane domain) and in the region between the transmembrane domains (11 and 27% identity, respectively), whereas a high degree of identity (87%) was detected in the C-terminal MSC domains of these proteins (Fig. 1A). Thus, we suggest that MAN1 and LEM2 comprise a subfamily within the LEM-domain proteins characterized by the presence of the MSC domain. Interestingly, Ce-LEM2, which has originally been termed Ce-MAN1 (Lee et al., 2000), is more homologous in the N-terminus and in the region between the transmembrane domains to hLEM2 than to hMAN1, and also lacks the C-terminal RNA recognition motif of MAN1. Therefore, we concur that the protein encoded by Ce-LEM2 is orthologous to human LEM2 (Mansharamani and Wilson, 2005), not MAN1, as originally thought (Lee et al., 2000). By contrast, a highly related protein from *Drosophila* (dmCG3167, Fig. 1A) seems to be a MAN1 rather than a LEM2 orthologue.

Phylogenetic analysis led us to speculate that LEM2 and MAN1 have a common ancestor in evolution (Fig. 1B). Intriguingly, a domain organization related to that of the predicted ancestor was also detected in yeast Src1p-related proteins. Src1p contains an N-terminal SAP domain, which by structural criteria might be similar to the LEM domain, two transmembrane domains and the conserved C-terminal MSC domain (Fig. 1B) (Mans et al., 2004). Thus, the LEM motif might have been derived from the SAP motif, and proteins containing the SAP/LEM and MSC domains might represent an evolutionarily conserved LEM2 protein family. In addition, a group of MAN1-type proteins, characterized by the presence of an additional C-terminal RRM domain (yellow), might have evolved from LEM2 ancestors.

LEM2 is an ubiquitously expressed INM protein

Having identified LEM2 as a novel member of the LEM protein family we wished to analyse the expression pattern and biochemical properties of the protein. Semiquantitative RT-PCR analysis revealed that LEM2 is ubiquitously expressed at comparable levels in all human and mouse tissues tested (Fig. 1D). Furthermore, LEM2 transcripts were also detectable throughout later stages of mouse development (dpc 12, 14, 16, 18; data not shown). These data are consistent with the previously reported ubiquitous expression of Ce-LEM2 (Gruenbaum et al., 2002).

In order to determine the subcellular localization of LEM2 protein, we stably expressed V5-tagged hLEM2 in various cell types. Confocal immunofluorescence microscopy of stable HeLa clones showed that the expressed LEM2 localized exclusively to the NE, giving rise to a typical rim-like staining of the nuclear periphery (Fig. 2A). Identical data were obtained in COS7 and MDCK cells, and in human and mouse primary skin fibroblasts (data not shown). In order to distinguish localization of LEM2 in the INM versus ONM, we permeabilized cells with digitonin, which selectively disrupts the plasma membrane leaving the NE membranes intact (Adam et al., 1990). Antibodies to the C-terminal V5 tag of hLEM2 and to the nucleoplasmic protein LAP2α did not detect the proteins in digitonin-treated interphase cells in the nucleus; by contrast, in mitotic cells, in which the nuclear membrane was disassembled, LEM2-V5 and LAP2α were visible in the cytoplasm (arrows, Fig. 2B). A weak LEM2 staining in digitonin-treated interphase cells in the cytoplasm might represent a small fraction of LEM2-V5 in the ER. Since LEM2 in the NE was not accessible for

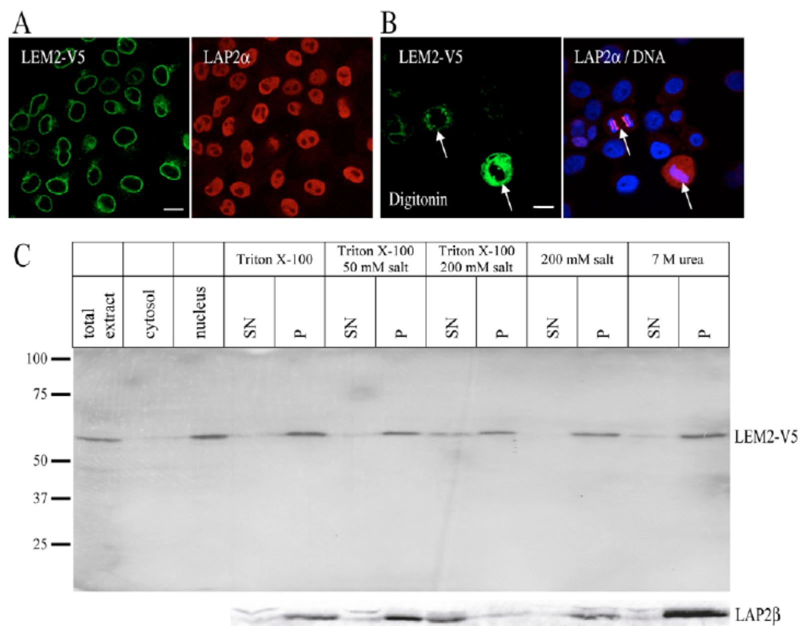


Fig. 2. Human LEM2 is a lamina protein of the INM. HeLa cells stably expressing V5-tagged hLEM2 were processed for immunofluorescence using anti-V5 (green) and anti-LAP2α (red) antibodies. DNA was stained with Hoechst (blue). Cells were either extracted with (A) Triton X-100 or (B) digitonin after fixation. Arrows point to mitotic cells, which serve as positive control for antibody reactivity. Bars, 10 μm. (C) Subcellular fractionation of lysates of stable HeLa clones expressing V5-tagged hLEM2. Nuclei were extracted with Triton X-100 and/or salt, or with 7M urea, and soluble supernatant and insoluble pellet fractions were subjected to western blot analysis using anti-V5 and anti-LAP2β antibodies. Molecular weights in kDa are indicated on the left.

Journal of Cell Science

antibodies in digitonin-treated interphase cells, we concluded that the LEM2 C-terminus is located in the nucleoplasm, implying that hLEM2 is a constituent of the INM.

To test whether hLEM2 is indeed a transmembrane protein and a component of the nuclear lamina, isolated nuclei of stable HeLa clones were extracted in buffers containing urea or non-ionic detergent, and the distribution of hLEM2 and of the well-characterized INM protein LAP2β (Foisner and Gerace, 1993) between soluble and insoluble fractions was analysed by immunoblotting (Fig. 2C). The observed M_r of LEM2-V5 of 60×10^3 was close to the calculated M_r (58×10^3). Similar to LAP2β, LEM2 remained insoluble in buffers containing either 200 mM salt or Triton X-100 and low salt concentration (50 mM), or 8M urea. Only treatment with detergent and medium salt concentration buffer (1% Triton/200 mM NaCl) solubilized a significant portion of LEM2 (Fig. 2C). Thus, by these biochemical criteria, LEM2 is a transmembrane protein of the INM associated with the nucleoskeletal-lamina network, similar to LAP2β. In support of this model, Schirmer and Gerace have detected rat LEM2 (NP_666187.1) in the nuclear membrane fraction by a subtractive proteomics approach (Schirmer et al., 2003).

During mitosis, when the nucleus is disintegrated and reassembled after sister chromatid separation, LEM2 behaved similarly to other LEM-domain-containing membrane proteins. From prophase to prometaphase, identified by the presence of phosphorylated histone H3 (Juan et al., 1998), the nuclear membrane was progressively disintegrated and LEM2 diffused into the cytoplasm, where it remained until telophase (Fig. 3A). Similar to emerin and LAP2β (Dechat et al., 2004; Haraguchi et al., 2001), LEM2 re-localized to the NE only at later stages of assembly, clearly after LAP2α was accumulated in chromatin-associated core regions and after LBR was detectable at peripheral regions of decondensing chromosomes (Fig. 3B). Altogether, based on its subcellular localization in the interphase nucleus, its dynamic behaviour during mitosis and its biochemical properties, LEM2 is indistinguishable from other integral nuclear membrane proteins of the LEM-domain family.

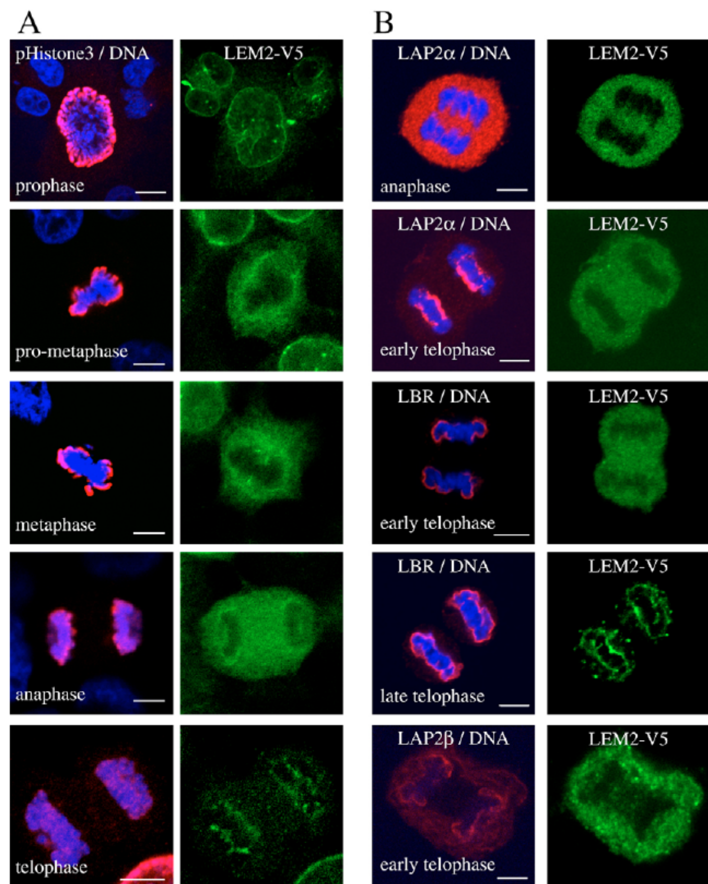


Fig. 3. Dynamics of LEM2 during the cell cycle. (A) HeLa cells stably expressing hLEM2 were processed for indirect immunofluorescence using anti-phospho-histone-H3 antibody (pHistone3; red) and anti-V5 antibody (green). DNA was stained using Hoechst dye (blue). Representative images of defined cell-cycle stages are shown. (B) Cells at anaphase or different telophase stages, were stained for exogenous LEM2 with anti-V5 antibody (green) and for either LAP2α, LBR or LAP2β (red). DNA was stained with Hoechst (blue). Bars, 10 μm in (A) and 5 μm in (B).

NE localization of hLEM2 requires A-type lamins at the NE

Extraction studies indicated that hLEM2 is associated with the lamina-nucleoskeleton scaffold (see above). Furthermore, in *C. elegans*, Ce-LEM2 localized to the NE in a lamin-dependent manner (Liu et al., 2003), and in mammals the LEM-domain protein emerin requires A-type lamins for NE targeting (Sullivan et al., 1999; Vaughan et al., 2001). To explore whether NE localization of hLEM2 also depends on A-type lamins, we analysed the localization of hLEM2 in MEFs. In wild-type MEFs, LEM2-V5 preferentially localized to the NE (Fig. 4A); however, in *Lmna*$^{-/-}$ MEFs, LEM2-V5 was distributed throughout the ER (Fig. 4B). Expression of GFP-lamin A in *Lmna*$^{-/-}$ MEFs, which localized to the NE, restored the predominantly rim-like localization of hLEM2-V5 at the NE (Fig. 4C), suggesting that lamin A is required for retention of LEM2 at the INM. To test this model further, we disrupted the distribution of endogenous A-type lamins in LEM2-V5-expressing HeLa cells by introducing a headless *Xenopus* lamin mutant, GFP-xLaminB1Δ2+, which accumulated in nucleoplasmic aggregates and caused the redistribution of endogenous A-type lamins from the peripheral lamina to these aggregates (Dechat et al., 2000a; Izumi et al., 2000). In cells

Journal of Cell Science

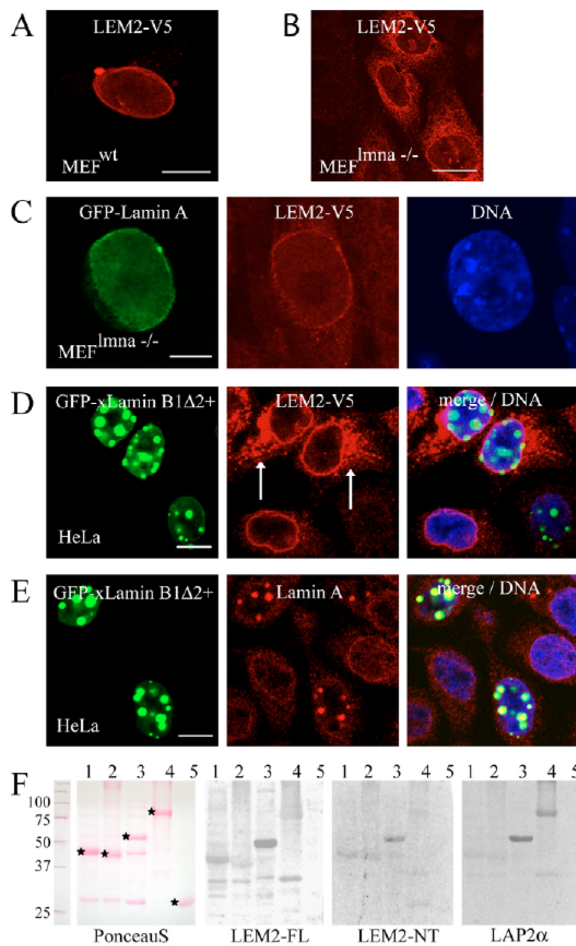


Fig. 4. Localization of LEM2 at the NE depends on the presence of A-type lamins. (A-C) Mouse embryonic fibroblasts (MEFs) from wild-type (A) or lamin A/C-knockout (B,C) mice were transfected with V5-tagged hLEM2 plasmids. In (C), cells expressing V5-tagged LEM2 were co-transfected with GFP-tagged pre-lamin A (GFP-LaminA) plasmid and were stained for LEM2 using anti-V5 antibody (A-D, red) and for DNA with Hoechst dye (C-E, blue). Ectopically expressed lamin A was detected by GFP fluorescence (C, green). (D,E) HeLa cells stably expressing V5-tagged hLEM2 were transfected with a construct encoding a GFP-tagged version of a dominant-negative *Xenopus* Lamin B1 (GFP-xLaminB1Δ2+; green) and stained either for LEM2 using anti-V5 antibody (D, red) or endogenous lamin A (E, red). Arrows depict mislocalization of LEM2 to the ER in cells expressing the dominant-negative lamin B mutant. Note, that V5-tagged LEM2 decorates only the nuclear rim in untransfected cells. Bars, 10 μm. (F) Recombinant GST alone (lanes 5), LAP2α (lanes 4) and GST-lamin C head domain (lanes 1), rod domain (lanes 2) and tail domain (lanes 3) were transblotted to nitrocellulose membranes. PonceauS staining detects recombinant proteins (asterisks). Membranes were probed with ^{35}S-labelled full-length LEM2 (LEM2-FL), the N-terminus of LEM2 (LEM2-NT) and LAP2α, and bound proteins were detected by autoradiography.

containing nuclear aggregates of mutated lamins, a significant fraction of hLEM2 localized to the presumed ER (Fig. 4D, arrows) whereas, in untransfected cells, hLEM2 was localized predominantly at the NE. As the mutated lamins clearly caused a mislocalization of endogenous lamin A/C in these cells (Fig. 4E), this finding is consistent with the hypothesis that loss of lamin A/C at the NE destabilizes the anchorage of LEM2 at the INM and causes its diffusion into the ER.

To test whether hLEM2 associates with A-type lamins, we performed blot overlay assays. ^{35}S-labelled full-length hLEM2 (FL) or an N-terminal hLEM2 fragment (NT) or LAP2α were overlaid onto immobilized GST-tagged lamin C fragments comprising the lamin C head, rod or tail domains, and onto immobilized LAP2α (Dechat et al., 2000a). Similar to the known lamin C-binding protein LAP2α, hLEM2 strongly bound to the C-terminus of lamin C (Fig. 4F, lane 3). The lamin C fragments containing the lamin C head (lane 1) or rod (lane 2) or LAP2α (lane 4) or GST alone (lane 5) did not bind hLEM2 above background level. The N-terminus of hLEM2 also bound to the lamin C C-terminus, although the interaction was weaker than those of full-length LEM2 (Fig. 4F). Altogether, our data strongly support a role of A-type lamins in targeting and stabilizing hLEM2 at the NE by a direct interaction.

The N-terminus of hLEM2 is required for retention at the INM

To determine the domain(s) of hLEM2 responsible for NE targeting and retention, we investigated the localization of hLEM2 fragments with a C-terminal V5 tag in HeLa cells. Whereas fragments lacking the C-terminus, including the MSC and the second transmembrane region (Fig. 5, LEM2ΔCT), still localized to the NE in a continuous rim, fragments missing nearly the complete N-terminus were no longer retained at the NE and localized mostly to the ER (LEM2ΔLEM+NT). This suggested that the N-terminus is essential for NE retention of LEM2. As LEM2 fragments missing a ~70aa region (aa 130-203) within the N-terminus close to the first transmembrane domain (LEM2ΔNT) behaved like wild-type protein, we concluded that the potential N-terminal nuclear retention signal is located upstream of position 130. To test whether the N-terminal retention signal is sufficient for LEM2 targeting, we expressed the N-terminus without the transmembrane domains and the C-terminus (LEM2-NT). This fragment localized to the nucleus but did not accumulate at the NE. Thus, the N-terminus is sufficient for nuclear targeting but not for targeting to the INM. The latter process requires an additional transmembrane region. This observation is consistent with previous studies showing that the localization of INM proteins to the nuclear periphery requires both a transmembrane domain and a retention signal mediating binding to a nuclear component (Holmer and Worman, 2001). In order to test whether the retention signal might reside within the LEM domain, which could retain LEM2 in the INM by binding to the chromatin protein BAF, we expressed the N-terminal 74 aa fragment of LEM2 containing the entire LEM motif (LEM2-LEM). However LEM2-LEM localized throughout the cell, indicating that the LEM domain of LEM2 is not sufficient for nuclear retention. On the basis of our observation that NE targeting of

LEM2 is dependent on the presence of A-type lamins, we reasoned that the retention domain within the N-terminus of LEM2 might mediate the association with lamin A/C complexes. To test this possibility, we expressed the LEM2 N-terminus in *Lmna*$^{-/-}$ MEFs. In contrast to wild-type HeLa cells, LEM2-NT did not accumulate in the nucleus in lamin A-deficient cells (Fig. 5, lower right panel). Taking all data together, targeting of LEM2 to the NE requires both a transmembrane domain and an N-terminal retention signal located between residues 74-130, which mediates association with lamin A/C complexes.

High-level overexpression of LEM2 affects NE structure

Low-to-moderate stable expression of hLEM2-V5 in several cell lines resulted in a smooth and continuous distribution of the protein at the nuclear periphery, which is typical for integral INM proteins (see Fig. 2A). If the protein was expressed at high levels (ratio of ectopic to endogenous LEM mRNA >2) in stable cell lines (e.g. HeLa and C2C12 myoblasts), it accumulated in discrete, patchy structures apparently localizing in the nuclear interior (Fig. 6A). However, in z-stacks or optical xz sections through these cells by confocal microscopy, all LEM2 structures were located at the nuclear periphery (Fig. 6B), and transmission electron microscopy resolved numerous finger-type intrusions of the nuclear membrane(s) in these cells (Fig. 6C). The LEM2 patches were often arranged in a regular pattern along the entire NE and varied in size (Fig. 6A,B). The LEM2 patches were not stained in digitonin-extracted cells (data not

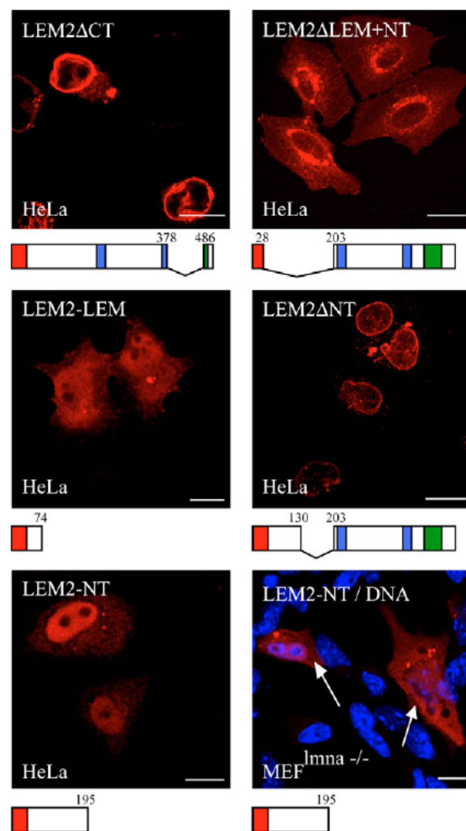


Fig. 5. The N-terminus of LEM2 is essential and sufficient for lamin A-mediated nuclear targeting. Confocal immunofluorescence images of HeLa cells or *Lmna*$^{-/-}$ MEFs transiently expressing different V5-tagged truncation mutants of hLEM2, as indicated in the schematic drawing below each image. The LEM domain is shown in red, transmembrane domains in blue, and MSC domain in green. Numbers depict aa that border each deletion. LEM2 polypeptides were detected by immunofluorescence using anti-V5 antibody (red). DNA was stained with Hoechst (blue). Arrows in the lower right image mark cytoplasmic staining of LEM2-NT in *Lmna*$^{-/-}$ MEFs. Bars, 10 μm.

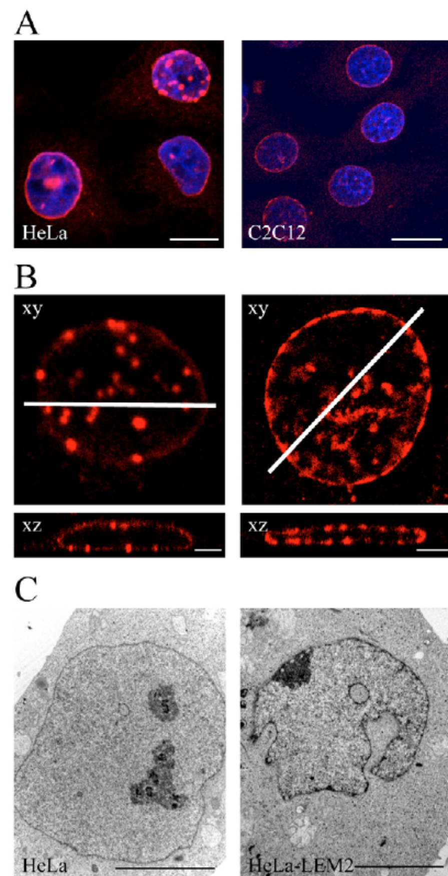


Fig. 6. Overexpressed LEM2 accumulates in peripheral patches. (A) HeLa or C2C12 cells expressing high levels of LEM2-V5 were processed for indirect immunofluorescence microscopy using anti-V5 antibody (red) and DNA was stained with Hoechst dye (blue). Bars, 10 μm. (B) Confocal images of cells in xy and xz optical sections. White line indicates location of xz section. Bars, 2 μm. (C) Transmission electron microscopic images of thin sections of embedded wild-type HeLa cells (left) or hLEM2-overexpressing HeLa cells (right). Bars, 5 μm.

Journal of Cell Science

shown, see also Fig. 2), suggesting that they are located at the INM.

To test whether formation of these LEM2 clusters can affect the distribution of other INM or lamina proteins, we analysed the localization of various NE proteins in transfected versus untransfected cells by double immunofluorescence microscopy. Lamin B1 (data not shown) and the lamin B-binding partner LBR (Fig. 7) did not significantly accumulate in LEM2 clusters. By contrast, A-type lamins were partially enriched in LEM2 patches, although they still localized throughout the entire NE (Fig. 7, arrowheads). Furthermore, emerin, which interacts with lamin A (Clements et al., 2000; Holaska et al., 2003), and BAF, which might be associated with the LEM domains of LEM2 and/or emerin, showed the most dramatic reorganizations to LEM2 clusters at the NE (Fig. 7). Therefore, we concluded that highly overexpressed LEM2 accumulated in patches in the INM and recruited A-type lamins and A-type lamin-binding proteins, whereas B-type lamins and their interaction partners (LBR) were not affected. These results indicate that LEM2 is associated with lamin A/C structures and support the model of a lamin A/C-dependent stabilization and retention of LEM2 at the NE shown above. Interestingly, overexpressed hMAN1-V5 did not accumulate in patches, but coexpression of MAN1-V5 and LEM2-GFP caused accumulation of both proteins in clusters, indicating that LEM2 can also recruit MAN1.

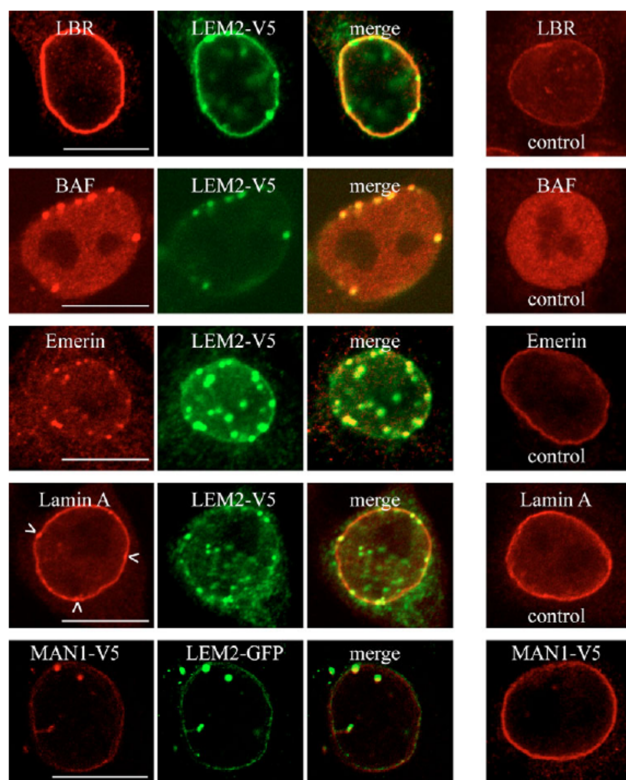


Fig. 7. A subset of NE proteins is recruited to LEM2 patches. HeLa cells constitutively expressing high levels of V5-tagged hLEM2 were processed for immunofluorescence microscopy using anti-V5 antibody (green) and antibodies to different NE proteins (red). Bottom row shows images of HeLa cells stably expressing V5-tagged MAN1 (red) and co-transfected with human LEM2-GFP (green)-encoding plasmid. Images on the right show untransfected HeLa cells stained for the NE proteins indicated (control; red) or cells expressing V5-tagged MAN1 alone (bottom row). Arrowheads in left image (row 4) depicts lamin A patches. Bars, 10 μm.

LEM2 overexpression induced formation of tubular structures between nuclei

Unlike untransfected control cells, a significant number (up to 10%) of LEM2-overexpressing cells contained tubular structures between nuclei of adjacent cells. These structures, which also contained LEM2, were up to 30 μm long and originated frequently from LEM2 patches at the NE (Fig. 8B, arrows). Double staining of these cells for actin (Fig. 8A) revealed that the tubular connections occur between individual cells that have completed cytokinesis. Furthermore, the tubular structures seem to be stable and can be maintained for a long time, as we detected up to four interconnected cells (Fig. 8F). Regarding the biogenesis of these tubular structures, it seems very likely that they are formed during NE assembly in telophase and G1. LEM2-containing tubular structures extend from LEM2 patches on chromatin in telophase and increase in length during progression to G1 phase (Fig. 8E, arrows). The formation of the tubular connections between adjacent cells apparently did not affect cell-cycle progression and subsequent cell divisions. Connected interphase cells contain phospho-histone 3, a marker for dividing cells (Fig. 8C), and cell pairs in late telophase/G1 phase can be linked to large interphase cells (Fig. 8D), indicating that cells with tubular connections can divide normally.

Double immunofluorescence microscopy using antibodies to different lamina proteins revealed that, similar to the LEM2-containing patches at the NE (Fig. 7), the tubular structures between the nuclei also contained lamin A, emerin and BAF (Fig. 9), whereas lamin B1 and LBR were hardly or not at all detectable (Fig. 9). Because the structures between nuclei contained several nuclear membrane proteins, they most probably represent membrane structures continuous with the NEs of connected nuclei. However, nuclear pores (Fig. 9, NUP62) and the ER protein α-calnexin were absent. Furthermore, cytoskeletal proteins such as tubulin (Fig. 9) or actin (Fig. 8) were not detectable in the connecting tubules. We propose that the recruitment of A-type lamins and lamin A-associated proteins to overexpressed LEM2 causes abnormalities of the NE, leading to the formation of extra membrane sheets extending from the chromatin-attached nuclear membranes.

Discussion

LEM2 and MAN1 comprise a subfamily within the LEM family that is highly conserved in evolution

In this study, we characterized a novel member of the LEM-domain protein family, which we named LEM2 according to a previously suggested nomenclature

Journal of Cell Science

(Lee et al., 2000). Extensive computational analyses defined LEM2 and MAN1 as a subfamily characterized by a common domain organization, consisting of an N-terminal LEM motif, two transmembrane domains and a highly conserved C-terminal MSC domain. MAN1 differs from LEM2 proteins by the presence of an additional RRM domain at the extreme C-terminus. On the basis of these criteria, Ce-LEM2, which has been described previously as a MAN1 orthologue in *C. elegans* (Lee et al., 2000), is a LEM2-type protein. Intriguingly, structure analyses of the LEM domain have indicated a homology with domains found in bacterial proteins, suggesting a common ancestor in evolution (Mans et al., 2004). According to this hypothesis, a helix-extension-helix motif found in the yeast protein Src1p might represent a common ancestor of both the LEM domain and the highly related SAP motif (Aravind and Koonin, 2000). Yeast Src1p might thus represent an ancestral LEM2-type protein as it harbours a LEM/SAP domain, two predicted transmembrane regions and the C-terminal MSC domain. Moreover, yeast Src1p might also share some functions with LEM2/MAN1 proteins, as a GFP-tagged Src1p has been localized to the yeast nuclear envelope, where it could be involved in sister chromatid segregation (Rodriguez-Navarro et al., 2002).

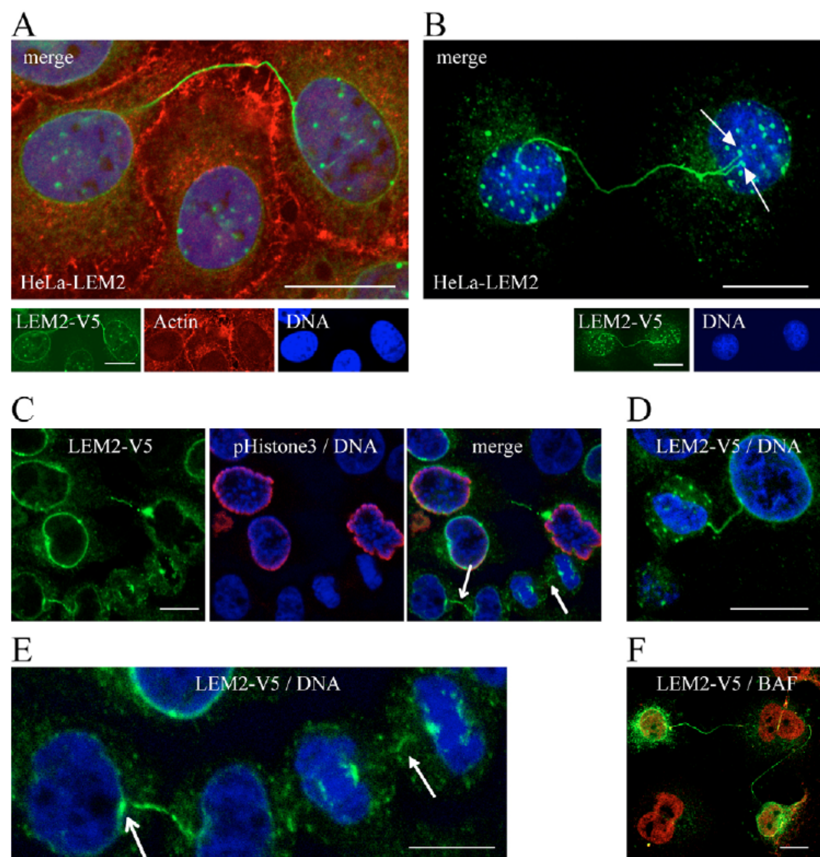


Fig. 8. LEM2-induced tubules are stable connections, persisting throughout several cell cycles. HeLa cells stably overexpressing V5-tagged hLEM2 were processed for immunofluorescence microscopy using antibodies to indicated proteins. Images depict LEM2 stained with anti-V5 (green) and actin (A, red) or phospho-histone H3 (C, red) or BAF (F, red), and DNA (A-E, blue). Arrows in (C) indicate cells shown at higher magnification in (E). Arrows in (E) depict LEM2 patches from which tubular structures emerge. Single colour images corresponding to the merged images in A and B are shown. Bars, 10 μm.

LEM2 is an INM protein linked to A-type lamin complexes

Our studies demonstrate that LEM2 is localized to the INM and is a genuine lamina protein, characterized by its co-fractionation with insoluble lamin structures upon treatment of nuclei with detergent and high salt (Foisner and Gerace, 1993). Unfortunately, our efforts to produce antibodies against N-terminal peptides remained unsuccessful and the characterization of mammalian LEM2 thus relies on ectopic expression data. Interestingly, we found that LEM2 required A-type lamins at the nuclear periphery for NE targeting. Lack of A-type lamins or disruption of endogenous lamin A structures caused a mislocalization of LEM2 to the ER. Thus, LEM2 can be considered as an A-type lamin-associated protein. Our in vitro binding data revealing an interaction of in-vitro-translated LEM2 with lamin C suggest direct interaction of these proteins, which is in accordance with various studies on Ce-LEM2 and human MAN1. First, Ce-LEM2, which is the only LEM2-type protein to date that has been analysed by biochemical and genetic means, also required lamins for its NE localization and has been shown to interact with Ce-lamin through the N-terminus (Ce-LEM2; aa 1-333) in blot overlay assays (Liu et al., 2003). Second, the N-terminus of MAN1 was found to interact in vitro with the globular tail domains of both A- and B-type lamins (Mansharamani and Wilson, 2005). In line with these findings, we also found that the domain mediating lamin A-dependent retention of LEM2 in the NE is localized in a region ~60 residues in length within the N-terminus of LEM2. A similar behaviour has been described for emerin, which binds to A-type lamins in vitro (Clements et al., 2000; Holaska et al., 2003) and requires A-type lamins for NE localization (Sullivan et al., 1999; Vaughan et al., 2001).

Journal of Cell Science

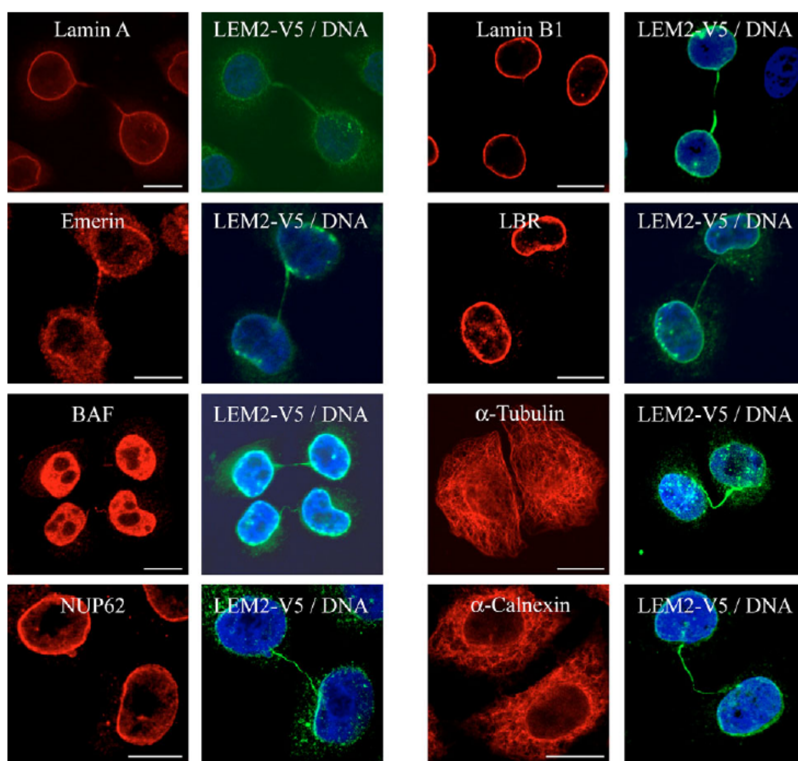


Fig. 9. LEM2-containing tubular structures between adjacent nuclei also contain lamin A/C-associated proteins. HeLa cells stably overexpressing V5-tagged hLEM2 were processed for immunofluorescence microscopy using antibodies to indicated proteins. Cells were stained for LEM2-V5 (green), DNA (blue), and either lamin A, emerin, BAF, NUP62, lamin B1, LBR, α-tubulin or α-calnexin (red). Bars, 10 μm.

endogenous LBR, accumulated in 'microdomains' within the NE (Makatsori et al., 2004). These observations suggest that the NE might be organized in different subdomains, each of which contains a specific subset of INM and lamina proteins.

In addition to LEM2 patches, we also observed tubular structures interconnecting nuclei of adjacent cells, which contained LEM2, lamin A, emerin, MAN1 and BAF, but rarely lamin B and LBR. Currently, the molecular details of how these membranous internuclear connections are formed are unknown. Analyses of the appearance of these structures in living cells suggested that they are formed at telophase emanating from LEM2 patches at chromatin, but we did not see anaphase bridges described in Ce-LEM2–Ce-emerin double-deficient or in Ce-BAF-deficient worms (Liu et al., 2003). Although we detected little chromatin in about 20% of these structures, we never found phosphorylated histone H3 as described in *C. elegans.* Excess LEM2 might disturb the assembly of A-type lamins and/or associated proteins, such as emerin and BAF, during post-metaphase NE reassembly. One could speculate that the internuclear bridges in LEM2-overexpressing cells are also caused by a defect in nuclear membrane assembly after sister chromatid separation, although the insertion of functional NPCs into these bridges was not observed. Interestingly, connected cells remained replication competent and re-entered mitosis normally. Altogether, the observed phenotypes upon overexpression of LEM2 imply functions of the protein in membrane assembly and in the dynamic NE organization during the cell cycle.

Potential functions of LEM2

High-level overexpression of LEM2 caused accumulation of the protein in patches at the NE and formation of NE invaginations, effects that were not observed with overexpressed human MAN1. Intriguingly, lamin A, emerin and BAF were, unlike lamin B1 and LBR, also reorganized to these structures, suggesting a role of LEM2 in the structural organization of a subset of NE components. As this subset of NE proteins included mostly proteins that have previously been detected in A-type lamin complexes, we propose that LEM2 is involved in the organization of A-type lamins within the NE. Given the fact that emerin and BAF seem to be recruited into these patch-like structures more efficiently than A-type lamins, it is tempting to speculate that these proteins might bind directly to LEM2 with high affinity. Whereas binding of BAF to LEM2 might be mediated by the LEM domain and a C-terminal region (Liu et al., 2003), binding of emerin might be mediated by a region in LEM2 related to MAN1, as an interaction between emerin and MAN1 has recently been demonstrated (Mansharamani et al., 2005). Interestingly, the LEM2 structures seem to be distinct from lamin B-containing protein complexes. Previous studies showed that overexpressed GFP-tagged LBR (Ellenberg et al., 1997), as well as

The properties of LEM2 presented here also make the protein an interesting candidate for involvement in laminopathy-type diseases (Burke and Stewart, 2002; Gotzmann, 2004; Gruenbaum et al., 2005; Hutchison and Worman, 2004; Mounkes and Stewart, 2004). First, LEM2 was found to associate with A-type lamin complexes. Disease-linked mutations in the *LMNA* gene could disrupt the potential function of this complex in nuclear and chromatin organization. Second, upon loss of lamin A, LEM2 behaved exactly like emerin, implying overlapping functions of these proteins also in vertebrates. As mutations in emerin were found to cause Emery-Dreifuss muscular dystrophy (EDMD) (Bione et al., 1994; Emery, 1987; Emery and Dreifuss, 1966; Manilal et al., 1996), LEM2 might be linked to similar diseases. In this context, it is important to note that only 40% of clinically

diagnosed EDMD cases are linked to mutations in either lamin A or emerin, whereas 60% of cases are probably caused by mutations in other NE components with similar functions as lamin A/C and emerin. Third, mutations in MAN1 have recently been linked to osteopoikilosis, Buschke-Ollendorf syndrome and melorheostosis characterized by increased bone density (Hellemans et al., 2004). However, it is likely that at least some of the clinical phenotypes detected in these diseases are related to the recently identified role of MAN1 as an antagonist of the pathways mediated by BMP, TGF-β or activin (Lin et al., 2004; Lin et al., 2005; Osada et al., 2003; Pan et al., 2005). So far, we have not been able to detect a similar antagonistic signalling activity for LEM2 (our unpublished data), most likely because LEM2 lacks the C-terminal RRM motif, known to mediate binding to the R-Smads (Osada et al., 2003; Pan et al., 2005). Nevertheless, signalling-independent functions of MAN1 might also contribute to the disease phenotype, in which case one would also expect clinical symptoms for loss of LEM2.

We thank K. Furukawa (Niigata University, Japan), E. Ivessa (Medical University of Vienna, Austria), H. Hermann (DKFZ, Heidelberg, Germany), D. Vaux (Sir William Dunn School of Pathology, Oxford, UK) and C. Stewart (NCI-Frederick, USA) for their generous gifts of reagents. This work was supported by a research grant from the Austrian Science Research Fund (FWF, P15312) to R.F. and by grant HSJ-2 from the 'Hochschuljubiläumsstiftung der Stadt Wien' to J.G.

References

Adam, S. A., Marr, R. S. and Gerace, L. (1990). Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* **111**, 807-816.

Aravind, L. and Koonin, E. V. (2000). SAP – a putative DNA-binding motif involved in chromosomal organization. *Trends Biochem. Sci.* **25**, 112-114.

Ashery-Padan, R., Ulitzur, N., Arbel, A., Goldberg, M., Weiss, A. M., Maus, N., Fisher, P. A. and Gruenbaum, Y. (1997). Localization and posttranslational modifications of otefin, a protein required for vesicle attachment to chromatin, during *Drosophila melanogaster* development. *Mol. Cell. Biol.* **17**, 4114-4123.

Bengtsson, L. and Wilson, K. L. (2004). Multiple and surprising new functions for emerin, a nuclear membrane protein. *Curr. Opin. Cell Biol.* **16**, 73-79.

Berger, R., Theodor, L., Shoham, J., Gokkel, E., Brok-Simoni, F., Avraham, K. B., Copeland, N. G., Jenkins, N. A., Rechavi, G. and Simon, A. J. (1996). The characterization and localization of the mouse thymopoietin/lamina-associated polypeptide 2 gene and its alternatively spliced products. *Genome Res.* **6**, 361-370.

Bione, S., Maestrini, E., Rivella, S., Mancini, M., Regis, S., Romeo, G. and Toniolo, D. (1994). Identification of a novel X-linked gene responsible for Emery-Dreifuss muscular dystrophy. *Nat. Genet.* **8**, 323-327.

Burge, C. and Karlin, S. (1997). Prediction of complete gene structures in human genomic DNA. *J. Mol. Biol.* **268**, 78-94.

Burke, B. and Stewart, C. L. (2002). Life at the edge: the nuclear envelope and human disease. *Nat. Rev. Mol. Cell. Biol.* **3**, 575-585.

Cai, M., Huang, Y., Ghirlando, R., Wilson, K. L., Craigie, R. and Clore, G. M. (2001). Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structures: one binds BAF and the other binds DNA. *EMBO J.* **20**, 4399-4407.

Clements, L., Manilal, S., Love, D. R. and Morris, G. E. (2000). Direct interaction between emerin and lamin A. *Biochem. Biophys. Res. Commun.* **267**, 709-714.

Cserzo, M., Eisenhaber, F., Eisenhaber, B. and Simon, I. (2004). TM or not TM: transmembrane protein prediction with low false positive rate using DAS-TMfilter. *BioInformatics* **20**, 136-137.

de Virgilio, M., Weninger, H. and Ivessa, N. E. (1998). Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J. Biol. Chem.* **273**, 9734-9743.

Dechat, T., Gotzmann, J., Stockinger, A., Harris, C. A., Talle, M. A., Siekierka, J. J. and Foisner, R. (1998). Detergent-salt resistance of LAP2alpha in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics. *EMBO J.* **17**, 4887-4902.

Dechat, T., Korbei, B., Vaughan, O. A., Vlcek, S., Hutchison, C. J. and Foisner, R. (2000a). Lamina-associated polypeptide 2alpha binds intranuclear A-type lamins. *J. Cell Sci.* **113**, 3473-3484.

Dechat, T., Vlcek, S. and Foisner, R. (2000b). Review: lamina-associated polypeptide 2 isoforms and related proteins in cell cycle-dependent nuclear structure dynamics. *J. Struct. Biol.* **129**, 335-345.

Dechat, T., Gajewski, A., Korbei, B., Gerlich, D., Daigle, N., Haraguchi, T., Furukawa, K., Ellenberg, J. and Foisner, R. (2004). LAP2{alpha} and BAF transiently localize to telomeres and specific regions on chromatin during nuclear assembly. *J. Cell Sci.* **117**, 6117-6128.

Dreger, C. K., Konig, A. R., Spring, H., Lichter, P. and Herrmann, H. (2002). Investigation of nuclear architecture with a domain-presenting expression system. *J. Struct. Biol.* **140**, 100-115.

Ellenberg, J., Siggia, E. D., Moreira, J. E., Smith, C. L., Presley, J. F., Worman, H. J. and Lippincott-Schwartz, J. (1997). Nuclear membrane dynamics and reassembly in living cells: targeting of an inner nuclear membrane protein in interphase and mitosis. *J. Cell Biol.* **138**, 1193-1206.

Emery, A. E. (1987). X-linked muscular dystrophy with early contractures and cardiomyopathy (Emery-Dreifuss type). *Clin. Genet.* **32**, 360-367.

Emery, A. E. and Dreifuss, F. E. (1966). Unusual type of benign x-linked muscular dystrophy. *J. Neurol. Neurosurg. Psychiatry* **29**, 338-342.

Foisner, R. (2001). Inner nuclear membrane proteins and the nuclear lamina. *J. Cell Sci.* **114**, 3791-3792.

Foisner, R. (2003). Cell cycle dynamics of the nuclear envelope. *ScientificWorldJournal* **3**, 1-20.

Foisner, R. and Gerace, L. (1993). Integral membrane proteins of the nuclear envelope interact with lamins and chromosomes, and binding is modulated by mitotic phosphorylation. *Cell* **73**, 1267-1279.

Furukawa, K. (1999). LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction. *J. Cell Sci.* **112**, 2485-2492.

Furukawa, K., Pante, N., Aebi, U. and Gerace, L. (1995). Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope. *EMBO J.* **14**, 1626-1636.

Furukawa, K., Fritze, C. E. and Gerace, L. (1998). The major nuclear envelope targeting domain of LAP2 coincides with its lamin binding region but is distinct from its chromatin interaction domain. *J. Biol. Chem.* **273**, 4213-4219.

Goldman, R. D., Gruenbaum, Y., Moir, R. D., Shumaker, D. K. and Spann, T. P. (2002). Nuclear lamins: building blocks of nuclear architecture. *Genes Dev.* **16**, 533-547.

Gotzmann, J., Vlcek, S. and Foisner, R. (2000). Caspase-mediated cleavage of the chromosome-binding domain of lamina-associated polypeptide 2 alpha. *J. Cell Sci.* **113**, 3769-3780.

Gotzmann, J., Huber, H., Thallinger, C., Wolschek, M., Jansen, B., Schulte-Hermann, R., Beug, H. and Mikulits, W. (2002). Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness. *J. Cell Sci.* **115**, 1189-1202.

Gotzmann, J. and Foisner, R. (2004). Lamins and emerin in muscular dystrophy: the nuclear envelope connection. In *Molecular Mechanisms of Muscular Dystrophies*, Chapter 12 (ed. S. J. Winder), pp. 1-18. Georgetown, Texas: Landes Biosciences.

Gruenbaum, Y., Lee, K. K., Liu, J., Cohen, M. and Wilson, K. L. (2002). The expression, lamin-dependent localization and RNAi depletion phenotype for emerin in C. elegans. *J. Cell Sci.* **115**, 923-929.

Gruenbaum, Y., Margalit, A., Goldman, R. D., Shumaker, D. K. and Wilson, K. L. (2005). The nuclear lamina comes of age. *Nat. Rev. Mol. Cell. Biol.* **6**, 21-31.

Haraguchi, T., Koujin, T., Segura-Totten, M., Lee, K. K., Matsuoka, Y., Yoneda, Y., Wilson, K. L. and Hiraoka, Y. (2001). BAF is required for emerin assembly into the reforming nuclear envelope. *J. Cell Sci.* **114**, 4575-4585.

Harris, C. A., Andryuk, P. J., Cline, S., Chan, H. K., Natarajan, A., Siekierka, J. J. and Goldstein, G. (1994). Three distinct human thymopoietins are derived from alternatively spliced mRNAs. *Proc. Natl. Acad. Sci. USA* **91**, 6283-6287.

Hellemans, J., Preobrazhenska, O., Willaert, A., Debeer, P., Verdonk, P. C., Costa, T., Janssens, K., Menten, B., Van Roy, N., Vermeulen, S. J. et

al. (2004). Loss-of-function mutations in LEMD3 result in osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis. *Nat. Genet.* **36**, 1213-1218.

Holaska, J. M., Lee, K. K., Kowalski, A. K. and Wilson, K. L. (2003). Transcriptional repressor germ cell-less (GCL) and barrier to autointegration factor (BAF) compete for binding to emerin in vitro. *J. Biol. Chem.* **278**, 6969-6975.

Holaska, J. M., Kowalski, A. K. and Wilson, K. L. (2004). Emerin caps the pointed end of actin filaments: evidence for an actin cortical network at the nuclear inner membrane. *PLoS Biol.* **2**, E231.

Holmer, L. and Worman, H. J. (2001). Inner nuclear membrane proteins: functions and targeting. *Cell. Mol. Life Sci.* **58**, 1741-1747.

Holt, I., Ostlund, C., Stewart, C. L., Man, N., Worman, H. J. and Morris, G. E. (2003). Effect of pathogenic mis-sense mutations in lamin A on its interaction with emerin in vivo. *J. Cell Sci.* **116**, 3027-3035.

Hutchison, C. J. and Worman, H. J. (2004). A-type lamins: guardians of the soma? *Nat. Cell Biol.* **6**, 1062-1067.

Izumi, M., Vaughan, O. A., Hutchison, C. J. and Gilbert, D. M. (2000). Head and/or CaaX domain deletions of lamin proteins disrupt preformed lamin A and C but not lamin B structure in mammalian cells. *Mol. Biol. Cell* **11**, 4323-4337.

Jayasinghe, S., Hristova, K. and White, S. H. (2001). MPtopo: A database of membrane protein topology. *Protein Sci.* **10**, 455-458.

Juan, G., Traganos, F., James, W. M., Ray, J. M., Roberge, M., Sauve, D. M., Anderson, H. and Darzynkiewicz, Z. (1998). Histone H3 phosphorylation and expression of cyclins A and B1 measured in individual cells during their progression through G2 and mitosis. *Cytometry* **32**, 71-77.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567-580.

Laguri, C., Gilquin, B., Wolff, N., Romi-Lebrun, R., Courchay, K., Callebaut, I., Worman, H. J. and Zinn-Justin, S. (2001). Structural characterization of the LEM motif common to three human inner nuclear membrane proteins. *Structure (Camb.)* **9**, 503-511.

Lee, K. K. and Wilson, K. L. (2004). All in the family: evidence for four new LEM-domain proteins Lem2 (NET-25), Lem3, Lem4 and Lem5 in the human genome. *Symp. Soc. Exp. Biol.* **56**, 329-339.

Lee, K. K., Gruenbaum, Y., Spann, P., Liu, J. and Wilson, K. L. (2000). *C. elegans* nuclear envelope proteins emerin, MAN1, lamin, and nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis. *Mol. Biol. Cell* **11**, 3089-3099.

Lee, K. K., Haraguchi, T., Lee, R. S., Koujin, T., Hiraoka, Y. and Wilson, K. L. (2001). Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. *J. Cell Sci.* **114**, 4567-4573.

Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P. and Bork, P. (2004). SMART 4.0: towards genomic data integration. *Nucleic Acids Res.* **32**, D142-D144.

Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I. S., Holmer, L., McBurney, M. W., Paulin-Levasseur, M. and Worman, H. J. (2000). MAN1, an inner nuclear membrane protein that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J. Biol. Chem.* **275**, 4840-4847.

Lin, F., Morrison, J. M., Wu, W. and Worman, H. J. (2005). MAN1, an integral protein of the inner nuclear membrane, binds Smad2 and Smad3 and antagonizes transforming growth factor-{beta} signaling. *Hum. Mol. Genet.* **14**, 437-445.

Liu, J., Lee, K. K., Segura-Totten, M., Neufeld, E., Wilson, K. L. and Gruenbaum, Y. (2003). MAN1 and emerin have overlapping function(s) essential for chromosome segregation and cell division in Caenorhabditis elegans. *Proc. Natl. Acad. Sci. USA* **100**, 4598-4603.

Makatsori, D., Kourmouli, N., Polioudaki, H., Shultz, L. D., McLean, K., Theodoropoulos, P. A., Singh, P. B. and Georgatos, S. D. (2004). The inner nuclear membrane protein lamin B receptor forms distinct microdomains and links epigenetically marked chromatin to the nuclear envelope. *J. Biol. Chem.* **279**, 25567-25573.

Manilal, S., Nguyen, T. M., Sewry, C. A. and Morris, G. E. (1996). The Emery-Dreifuss muscular dystrophy protein, emerin, is a nuclear membrane protein. *Hum. Mol. Genet.* **5**, 801-808.

Mans, B. J., Anantharaman, V., Aravind, L. and Koonin, E. V. (2004). Comparative genomics, evolution and origins of the nuclear envelope and nuclear pore complex. *Cell Cycle* **3**, 1612-1637.

Mansharamani, M. and Wilson, K. L. (2005). Nuclear membrane protein MAN1: Direct binding to emerin in vitro and two modes of binding to BAF. *J. Biol. Chem.* **280**, 13863-13870.

Markiewicz, E., Dechat, T., Foisner, R., Quinlan, R. A. and Hutchison, C. J. (2002). Lamin A/C binding protein LAP2alpha is required for nuclear anchorage of retinoblastoma protein. *Mol. Biol. Cell* **13**, 4401-4413.

Maske, C. P. and Vaux, D. J. (2004). CAAX-dependent modifications of the lamin proteins in the organization of the nuclear periphery. *Symp. Soc. Exp. Biol.* **56**, 317-328.

Mounkes, L. C. and Stewart, C. L. (2004). Aging and nuclear organization: lamins and progeria. *Curr. Opin. Cell Biol.* **16**, 322-327.

Muchir, A., Medioni, J., Laluc, M., Massart, C., Arimura, T., van der Kooi, A. J., Desguerre, I., Mayer, M., Ferrer, X., Briault, S. et al. (2004). Nuclear envelope alterations in fibroblasts from patients with muscular dystrophy, cardiomyopathy, and partial lipodystrophy carrying lamin A/C gene mutations. *Muscle Nerve* **30**, 444-450.

Nili, E., Cojocaru, G. S., Kalma, Y., Ginsberg, D., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Berger, R., Shaklai, S., Amariglio, N. et al. (2001). Nuclear membrane protein LAP2beta mediates transcriptional repression alone and together with its binding partner GCL (germ-cell-less). *J. Cell Sci.* **114**, 3297-3307.

Osada, S., Ohmori, S. Y. and Taira, M. (2003). XMAN1, an inner nuclear membrane protein, antagonizes BMP signaling by interacting with Smad1 in *Xenopus* embryos. *Development* **130**, 1783-1794.

Ostlund, C., Bonne, G., Schwartz, K. and Worman, H. J. (2001). Properties of lamin A mutants found in Emery-Dreifuss muscular dystrophy, cardiomyopathy and Dunnigan-type partial lipodystrophy. *J. Cell Sci.* **114**, 4435-4445.

Ovcharenko, I., Boffelli, D. and Loots, G. G. (2004). eShadow: a tool for comparing closely related sequences. *Genome Res.* **14**, 1191-1198.

Pan, D., Estevez-Salmeron, L. D., Stroschein, S. L., Zhu, X., He, J., Zhou, S. and Luo, K. (2005). The integral inner nuclear membrane protein MAN1 physically interacts with the R-Smad proteins to repress signaling by the TGFbeta superfamily of cytokines. *J. Biol. Chem.* **280**, 15992-16001.

Raharjo, W. H., Enarson, P., Sullivan, T., Stewart, C. L. and Burke, B. (2001). Nuclear envelope defects associated with LMNA mutations cause dilated cardiomyopathy and Emery-Dreifuss muscular dystrophy. *J. Cell Sci.* **114**, 4447-4457.

Rodriguez-Navarro, S., Igual, J. C. and Perez-Ortin, J. E. (2002). SRC1: an intron-containing yeast gene involved in sister chromatid segregation. *Yeast* **19**, 43-54.

Schirmer, E. C., Florens, L., Guan, T., Yates, J. R., 3rd. and Gerace, L. (2003). Nuclear membrane proteins with potential disease links found by subtractive proteomics. *Science* **301**, 1380-1382.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. USA* **95**, 5857-5864.

Segura-Totten, M. and Wilson, K. L. (2004). BAF: roles in chromatin, nuclear structure and retrovirus integration. *Trends Cell Biol.* **14**, 261-266.

Segura-Totten, M., Kowalski, A. K., Craigie, R. and Wilson, K. L. (2002). Barrier-to-autointegration factor: major roles in chromatin decondensation and nuclear assembly. *J. Cell Biol.* **158**, 475-485.

Shimi, T., Koujin, T., Segura-Totten, M., Wilson, K. L., Haraguchi, T. and Hiraoka, Y. (2004). Dynamic interaction between BAF and emerin revealed by FRAP, FLIP, and FRET analyses in living HeLa cells. *J. Struct. Biol.* **147**, 31-41.

Shumaker, D. K., Lee, K. K., Tanhehco, Y. C., Craigie, R. and Wilson, K. L. (2001). LAP2 binds to BAF.DNA complexes: requirement for the LEM domain and modulation by variable regions. *EMBO J.* **20**, 1754-1764.

Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C. L. and Burke, B. (1999). Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. *J. Cell Biol.* **147**, 913-920.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.

Vaughan, A., Alvarez-Reyes, M., Bridger, J. M., Broers, J. L., Ramaekers, F. C., Wehnert, M., Morris, G. E., Whitfield, W. G. F. and Hutchison, C. J. (2001). Both emerin and lamin C depend on lamin A for localization at the nuclear envelope. *J. Cell Sci.* **114**, 2577-2590.

Vergnes, L., Peterfy, M., Bergo, M. O., Young, S. G. and Reue, K. (2004). Lamin B1 is required for mouse development and nuclear integrity. *Proc. Natl. Acad. Sci. USA* **101**, 10428-10433.

Vlcek, S., Korbei, B. and Foisner, R. (2002). Distinct functions of the unique C terminus of LAP2alpha in cell proliferation and nuclear assembly. *J. Biol. Chem.* **277**, 18898-18907.

Wagner, N., Schmitt, J. and Krohne, G. (2004). Two novel LEM-domain

proteins are splice products of the annotated Drosophila melanogaster gene CG9424 (Bocksbeutel). *Eur. J. Cell Biol.* **82**, 605-616.

Worman, H. J., Yuan, J., Blobel, G. and Georgatos, S. D. (1988). A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA* **85**, 8531-8534.

Ye, Q. and Worman, H. J. (1994). Primary structure analysis and lamin B and DNA binding of human LBR, an integral protein of the nuclear envelope inner membrane. *J. Biol. Chem.* **269**, 11306-11311.

Zastrow, M. S., Vlcek, S. and Wilson, K. L. (2004). Proteins that bind A-type lamins: integrating isolated clues. *J. Cell Sci.* **117**, 979-987.

Journal of Cell Science

3.2.2 Localization and Phenotypic Consequences of Expressed LEM2 Protein Domains

The N-terminal portion of human LEM2 has previously been identified to interact directly with Lamin A/C thereby anchoring the protein at the INM (Brachner *et al.* 2005). During the past years increasing evidence has linked Lamin A/C to cell cycle control, such as the discovery of a nucleoplasmic complex of Lamin A/C and Retinoblastoma protein (pRB) (Markiewicz *et al.* 2002; Ozaki *et al.* 1994) a tumor suppressor and major cell cycle and differentiation regulator protein (Genovese *et al.* 2006). Furthermore, cells derived from lmna$^{-/-}$ mice were found to proliferate significantly faster than wild type control cells, an effect which was linked to decreased pRB stability (Johnson *et al.* 2004) and to the lack of the suppressive function of Lamin A on the transcription factor AP-1 (Activator Protein 1). Ivorra and colleagues reported that c-fos, together with c-jun, which form the functional AP-1 complex, is sequestered to the NE and thereby inactivated by interaction with Lamin A (Ivorra *et al.* 2006). Additionally, LAP2α as the only so far described nucleoplasmic LEM protein, was shown to reside in the complex with intranuclear Lamin A/C and pRB and was therefore suggested to function in the regulation of cell cycle progression and differentiation (Dorner *et al.* 2006; Dorner *et al.* 2007). Although controversial results regarding the proliferation phenotype of human and murine cells deficient for either Lamin A/C or LAP2α have been published, these data point towards a function of both nuclear proteins in controlling cell cycle progression (Pekovic *et al.* 2007). The structural conformation and function of intranuclear versus peripheral Lamin A/C is still unclear (Moir *et al.* 2000), but it was suggested that the distribution of Lamin A/C could influence cell cycle as well as differentiation (Markiewicz *et al.* 2005; Muralikrishna *et al.* 2001). Further on, an increased level of peripheral Lamin A/C was observed to be correlated with entry into senescence whereas the localization of a Lamin A/C subset to the nucleoplasm was intimately associated with a proliferative state (Dorner and Naetar, unpublished data). Following our previous finding that ectopically expressed LEM2 N-terminus is homogenously distributed within the nucleoplasm, likely through interaction with intranuclear Lamin A/C, we aimed at investigating the influence of LEM2 on Lamin A/C function regarding cell cycle regulation in more detail.

A second structural motif, proposed to form a winged-helix like DNA binding structure (Caputo *et al.* 2006), which has previously been identified within MAN1 C-terminus may also exist in LEM2. This domain, termed MSC motif, is highly conserved in primary sequence between LEM2 and MAN1 and was described as an evolutionary conserved motif by computational analyses (Brachner *et al.* 2005; Mans *et al.* 2004) (the MSC motif was recently included in the

"PFAM"-database: PF09402). Hence, we aimed at elucidating the putative role of this motif within the LEM2 protein. One prominent feature of LEM2 upon ectopic expression in cells was the formation of protein patches at the nuclear envelope, which were resolved as large membrane invaginations at the ultrastructural level. Analyzing various LEM2 deletion constructs revealed that this putative DNA-interacting domain was required for the formation of patches, pointing to a role of LEM2 in organizing the chromatin structure at the nuclear periphery.

Results

Distinct domains contribute to LEM2 localization at the nuclear envelope

In order to characterize LEM2's individual domains in more detail, we tested the localization pattern and the influence of stably expressed full-length LEM2 or various LEM2 fragments missing the LEM, MSC or both domains, on cell cycle control. Whereas HeLa cells overexpressing ectopic full-length LEM2 were readily generated, only few clones with low expression levels could be obtained for expressing LEM2 lacking the LEM domain or both LEM and MSC domain (LEM2ΔL and LEM2ΔLM respectively). Repeatedly, no clones with stable expression of LEM2ΔMSC (LEM2ΔM) were viable. This indicated that, unlike full-length LEM2, LEM2-fragments are toxic to cells. Expectedly, all transiently expressed fragments were targeted to the NE, though with different efficiencies and showed a prominent nuclear rim staining or peripheral patches in immunofluorescence microscopy (Fig. 19). Whereas LEM2ΔL showed the LEM2-typical patch formation at the NE (Brachner *et al.* 2005), LEM2ΔM and LEM2ΔLM were distributed homogenously along the nuclear rim barely exhibiting accumulation in patches at the NE. Interestingly, formation of patches is a specific feature of the LEM2 protein as overexpressed human MAN1 exhibited homogenous distribution along the NE, despite the fact that the MSC motif is highly conserved between both proteins (Fig. 19, lower panel).

Retention of LEM2ΔL at the NE was slightly and that of LEM2ΔLM significantly reduced as indicated by an increase of fluorescent signal in the ER and cytoplasm (Fig. 19). The formation of NE patches as well as the connecting tubes between nuclei of adjacent cells as observed previously upon overexpression of LEM2 (Brachner *et al.* 2005) was found to be strictly dependent on the MSC motif, while the LEM domain seemed to contribute only slightly to this property (Table 4). Constructs lacking the C-terminus neither formed patches nor links, and LEM2ΔLEM showed patch formation with reduced frequency. Furthermore, the insertion of the fragments into the membrane was essential for patch formation, as expression of a C-terminal fragment without the transmembrane domains did not form patches or links (data not shown). Further on we tested the recruitment of BAF to NE patches, which is a supposed binding partner

of LEM2, previously shown to co-localize with LEM2 patches (Brachner *et al.* 2005). As expected, constructs lacking the LEM domain, i.e. LEM2ΔL and LEM2ΔLM, did not recruit BAF (Fig. 20A).

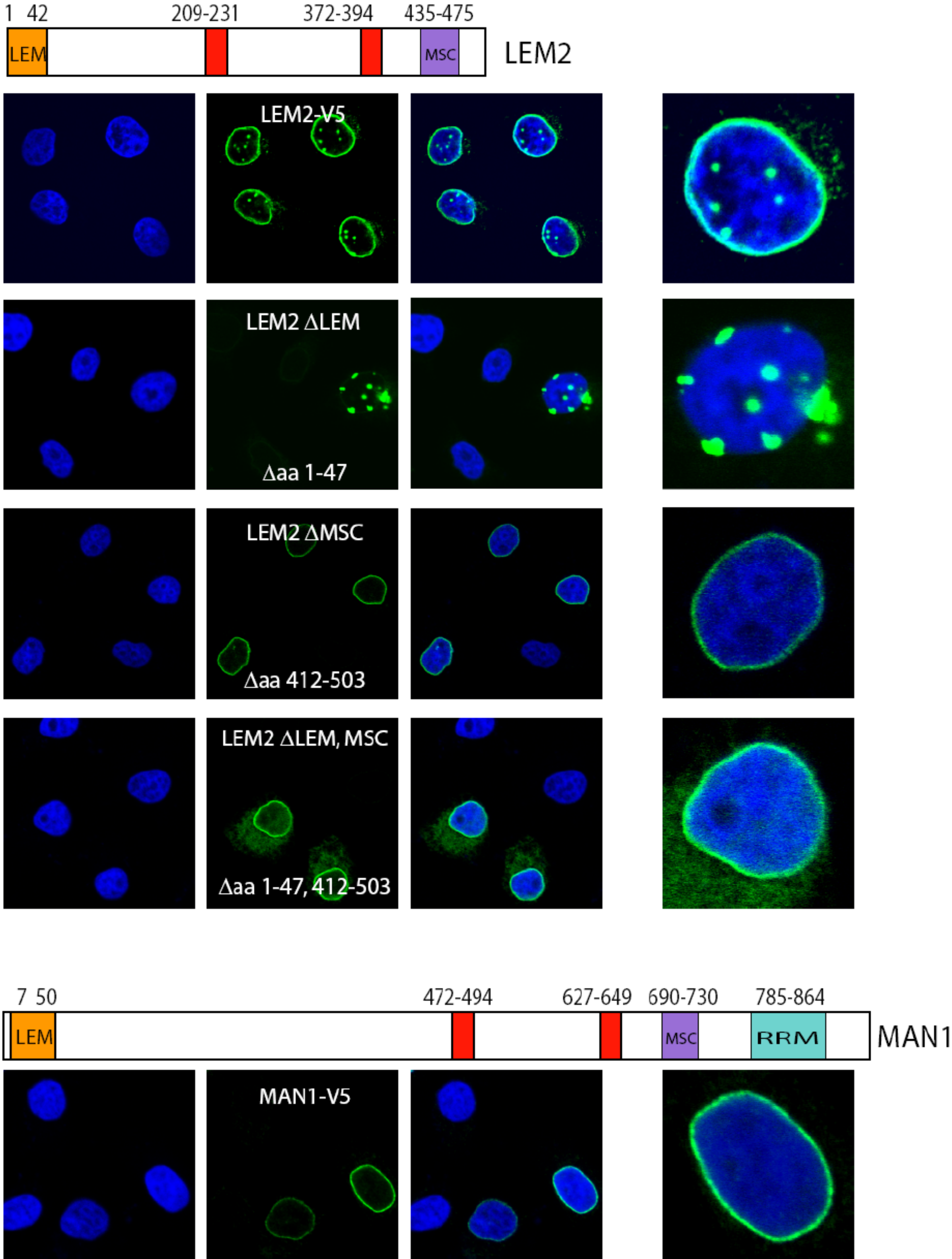


Figure 19: Immunofluorescence images of HeLa cells expressing LEM2-V5, MAN1-V5 or various human LEM2 deletion fragments. Schematic drawing of constructs: Orange box (LEM domain), red (transmembrane domain), violet (MSC motif), turquoise (RRM motif).

	NE patches	NE links
LEM2	+	+
LEM2ΔLEM	+, reduced frequency	+, reduced frequency
LEM2ΔMSC	–	–
LEM2ΔLEMΔMSC	–	–

Table 4: Occurrence of NE patches and links upon ectopic expression of LEM2 constructs.

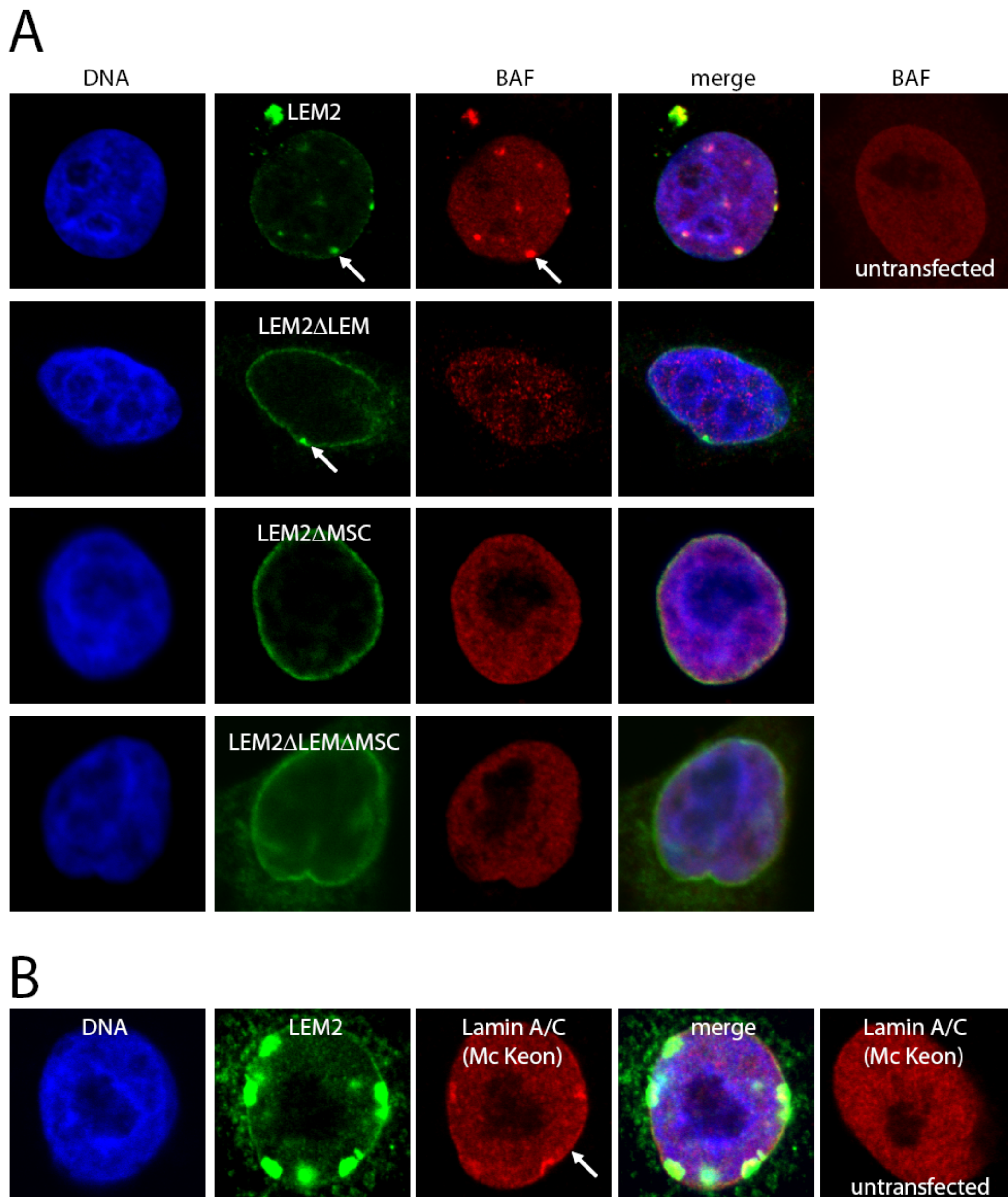


Figure 20: Immunofluorescence images of HeLa cells expressing LEM2-V5 or V5-tagged LEM2 deletion constructs. (A) HeLa cells expressing LEM2 constructs were co-stained for V5 (green), DNA (blue) and BAF (red). Arrows: Patches of LEM2 at the NE. (B) HeLa cells expressing full-length LEM2-V5 were stained for V5 (green), DNA (blue) and nucleoplasmic Lamin A/C (red). Arrow: Increased signal of Lamin A/C at the nuclear periphery.

These findings were further corroborated by *in vitro* binding assays performed in collaboration with Malini Mansharamani and Katherine Wilson (John Hopkins University School of Medicine, Baltimore, USA) (Fig. 21). Applying co-immunoprecipitation (Fig. 21A) and microtiter assays (Fig. 21B) it was shown that BAF bound directly to LEM2 containing the LEM-domain but not to LEM2ΔLEM or LEM2 C-terminus as it has been shown for MAN1 C-terminus (Mansharamani and Wilson 2005).

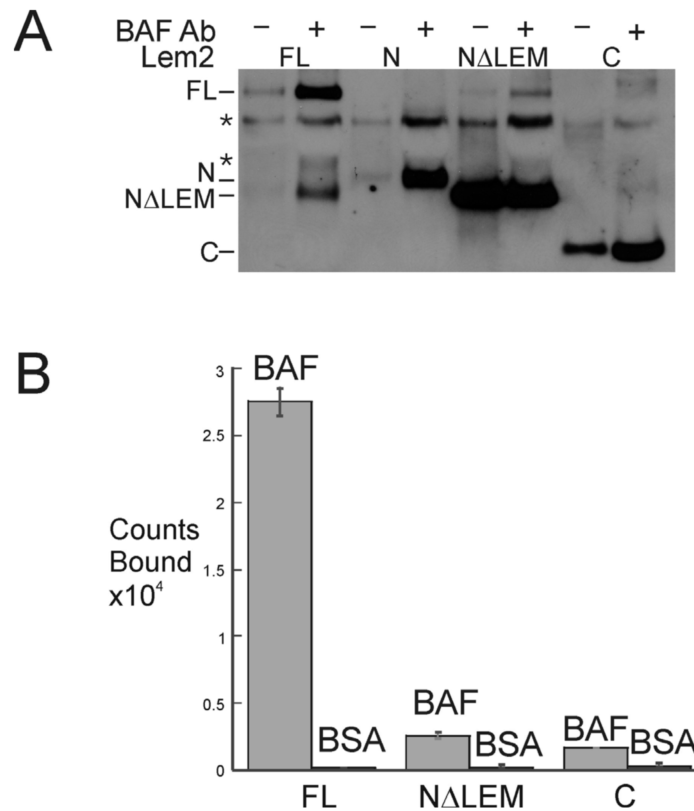


Figure 21: (A) Direct binding of BAF to LEM2 fragments tested by co-immunoprecipitation and microtiter assays. Western blot (upper panel) showing immunoprecipitation results. Recombinant LEM2 (FL), LEM2-N-terminus (N), LEM2-NT ΔLEM (NΔLEM) or LEM2-C-terminal (C) proteins were incubated with recombinant BAF in the presence (+) or absence (—) of antibodies against BAF. Immunoprecipitates were resolved by SDS-PAGE and western blotted with antibodies to V5 to detect LEM2 polypeptides. Asterisks (*) indicate nonspecific bands. (B) Microtiter assays. Recombinant purified BAF or BSA were immobilized in microtiter wells, incubated with ^{35}S-labeled probe protein LEM2-full length (FL), LEM2-NTΔLEM (NΔLEM) or LEM2-C fragment (C), then washed and quantified (n=3 triplicate sets). Bars indicate standard deviations. Experiment was carried out by M. Mansharamani & K. Wilson (Baltimore, USA).

In accordance with Ulbert and colleagues who reported that hLEM2 downregulation impairs cell cycle progression in HeLa and U2OS cells (Ulbert *et al.* 2006), we recognized different proliferation rates of HeLa cells stably expressing LEM2 fragments under standard cultivation conditions. Expression levels of ectopic proteins were determined by Western blot analysis (Fig. 22). Highest expression levels were observed for full-length LEM2. Interestingly, these cells also grew faster than untransfected HeLa. In contrast, LEM2ΔL and LEM2ΔLM were expressed at significantly lower levels and stable cells expressing these fragments reduced proliferation compared to control cells. Growth curve analyses with different clones were done to confirm these data. Three different single cell clones expressing full-length LEM2 proliferated significantly faster than untransfected HeLa cells, whereas one clone expressing LEM2ΔL and two out of three clones expressing LEM2ΔLM showed reduced proliferation (Fig. 23).

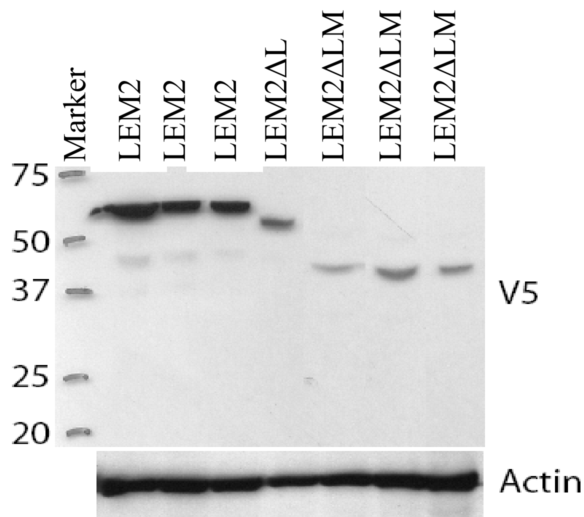


Figure 22: Western blot analysis of lysates prepared from HeLa single cell clones stably expressing V5-tagged full-length LEM2 or LEM2 deletion constructs.

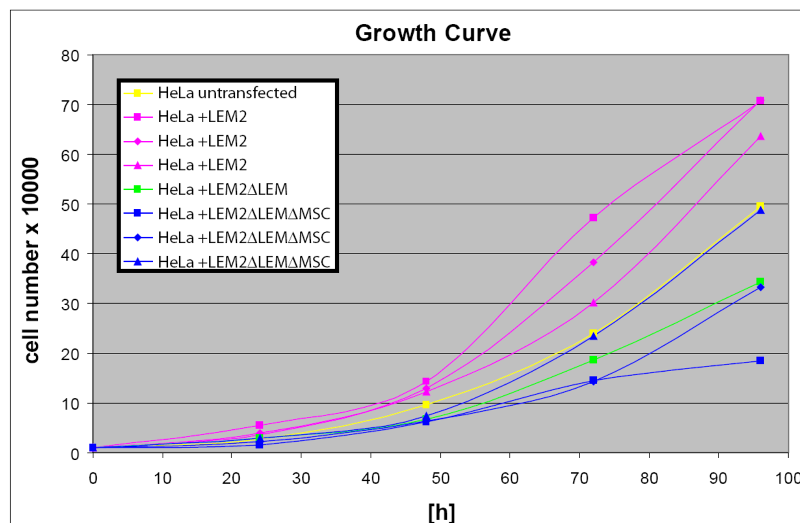


Figure 23: Growth curve of HeLa cells as well as different single cell clones stably overexpressing full-length LEM2, LEM2ΔL or LEM2ΔLM constructs.

Interestingly, overexpression of full-length LEM2 in cells caused a partial relocalization of nucleoplasmic Lamin A/C to the periphery (Fig. 20B), which may contribute to the observed influence on proliferation.

Expression of LEM2 N-terminal fragments recruits peripheral Lamin A/C to the nuclear interior and causes cell death

In our previous studies we defined a stretch of about 60 aa length within the LEM2 N-terminus to interact directly with A-type Lamins (Brachner *et al.* 2005). Ectopically expressed LEM2 N-terminal fragment lacking the transmembrane domain was localized primarily within the nucleoplasm in HeLa cells, while it localized to the nucleoplasm and cytoplasm in $lmna^{-/-}$ fibroblasts. Intriguingly, the amount of intranuclear Lamin A/C in cells expressing LEM2 N-terminal fragments (LEM2-NT) was slightly increased as compared to untransfected cells (Fig. 24, “Lamin A/C N18” and “Lamin A 133A2”). The increase in nucleoplasmic Lamin A/C was though quite subtle because specific staining for intranuclear lamins (Fig. 24, “McKeon”) did not show a clear difference between transfected and control cells. The localization and protein levels of Lamin B1, LAP2β, and LAP2α were not affected by LEM2-NT (Fig. 24). Generally, the distribution of LEM2-NT within the nucleus was uniform in the majority of cells. Intriguingly, BAF staining intensity was reduced in cells expressing LEM2-NT. This may be the result of epitope masking of nucleoplasmic BAF through interaction with nucleoplasmic LEM2-NT. Occasionally (in ~1% of transfected cells), however, we observed formation of patches of BAF at the NE in addition to reduced BAF staining in the nuclear interior, indicating that overexpression of LEM2-NT may also affect BAF localization (Fig. 25).

As our attempts to generate stable cell lines expressing LEM2-NT repeatedly failed and no mitotic figures could be found in cells expressing LEM2-NT transiently, we speculated that the recruitment of Lamin A/C to the nuclear interior by LEM2-NT might disturb pRB functions and cause an inhibitory effect on cell cycle progression. In order to determine the capability of LEM2-NT expressing cells to cycle we co-stained for the proliferation marker Ki-67. As cells expressing L2-NT were positive for Ki-67, we assumed that LEM2-NT expression did not cause quiescence or senescence (Fig. 26).

BrdU-labelling of cells revealed an increase in the relative number of S-phase cells in cultures expressing LEM2-NT compared to GFP and full-length LEM2 expressing controls (Fig. 27, “LEM2-NT”) (~45 versus 53% positive cells). It is unclear, however, whether this increase in S-phase cells is linked to the toxic effect of the LEM2-NT fragment. We speculate that the detrimental effect of LEM2 N-terminus on cell viability might be explained by induction of apoptosis or necrosis following activation of cell cycle checkpoints on impaired cell cycle regulation.

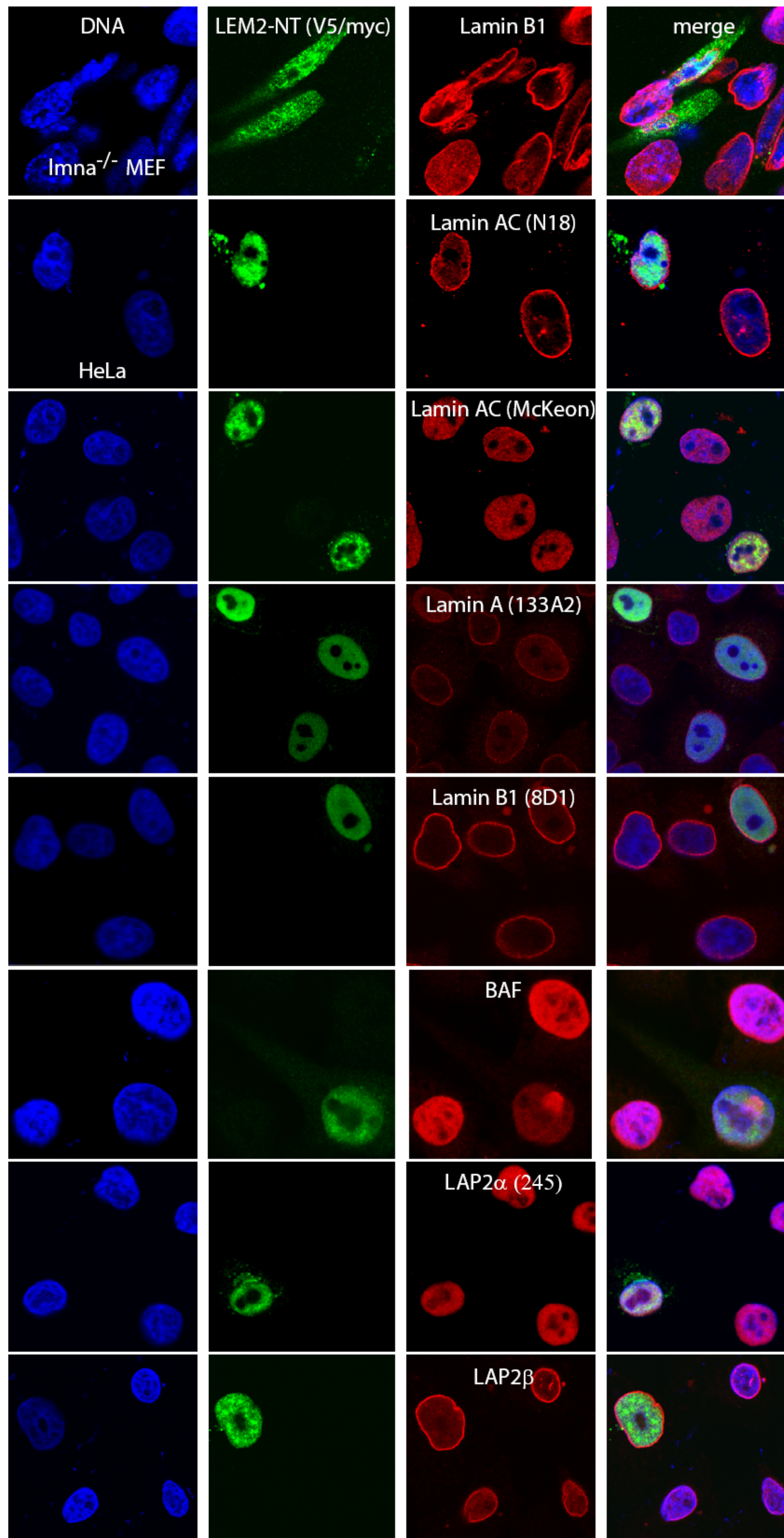


Figure 24: Immunofluorescence co-stainings of MEFs derived from Lamin A/C deficient mice and HeLa cells expressing myc- or V5 tagged LEM2 N-terminal fragments. Arrows: Lamin A recruited to the nuclear interior in transfected cells.

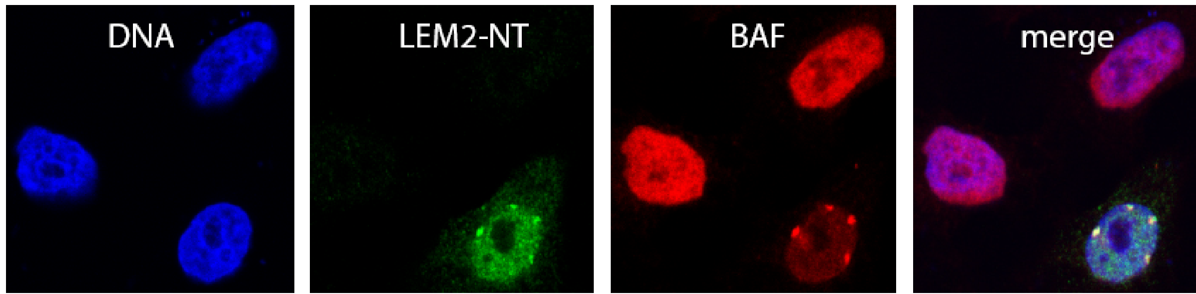


Figure 25: HeLa cells transiently expressing L2-NT were co-stained for BAF. In ~ 1% of transfected cells NE, patches of LEM2-NT and BAF were observed.

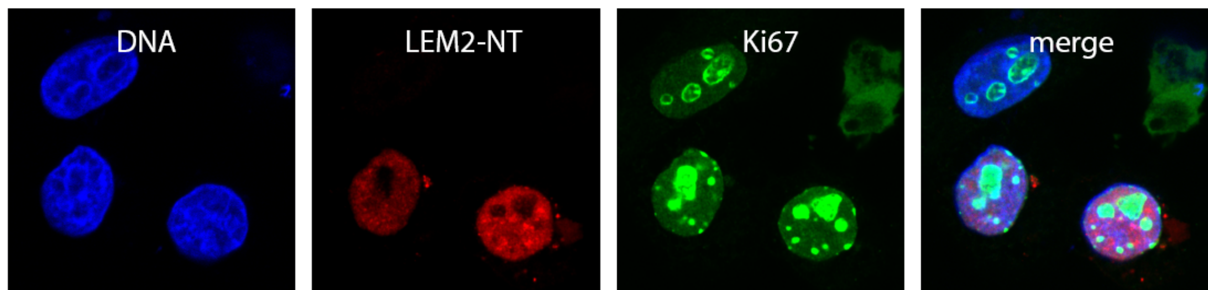


Figure 26: HeLa cells transiently expressing L2-NT were co-stained for Ki-67.

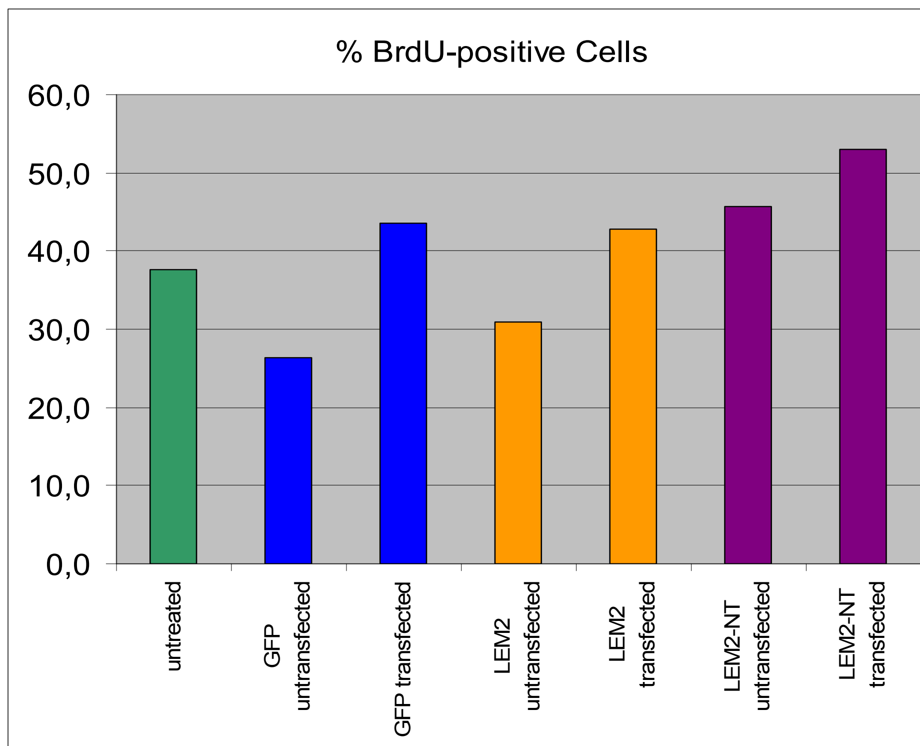


Figure 27: Quantification of BrdU-positive cells 24h after upon transfection with plasmids encoding GFP, LEM2 or LEM2-N-terminus (LEM2-NT) and a pulse-label of 30 minutes. Approximately 200 cells were evaluated for BrdU-label, untransfected cells of the same sample were counted as control for transfection and labeling procedure.

LEM2 N-terminal fragments affect localization of ectopic full-length LEM2 at the nuclear envelope

As Lamin A/C is required for localization of LEM2 at the NE (Brachner *et al.* 2005) we investigated the effects of LEM2-NT mediated Lamin A/C relocalization on the localization of full-length LEM2. Therefore, a myc-tagged LEM2-NT fragment was expressed in HeLa cells stably overexpressing full-length LEM2-V5. Interestingly, LEM2-V5 retention at the NE was significantly reduced in cells transfected with the LEM2-NT-myc construct (Fig. 28) indicating a competition of full-length LEM2 and LEM2-NT for binding sites at the nuclear periphery. This effect resembled the effect of a dominant Lamin B construct which aggregated peripheral Lamin A/C and caused displacement of LEM2-V5 from the NE (Brachner *et al.* 2005).

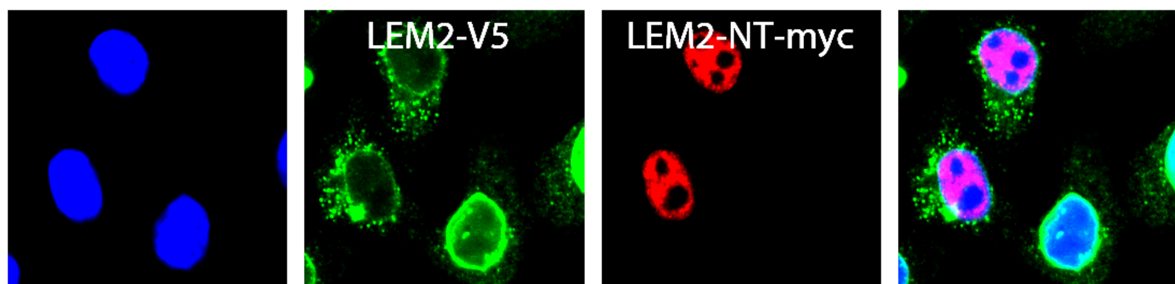


Figure 28: Immunofluorescence images of a stable HeLa cell line expressing full-length LEM2-V5 (green) and transiently expressing LEM2-NT-myc (red).

Furthermore, we were interested in the localization and phenotypical consequences of the LEM2 MSC domain. V5-, myc- and GFP-tagged C-terminal fragments of LEM2 (LEM2-CT) were expressed in HeLa cells, but neither V5- nor myc-tagged proteins were clearly detectable in the cells most likely due to toxic effects (data not shown).

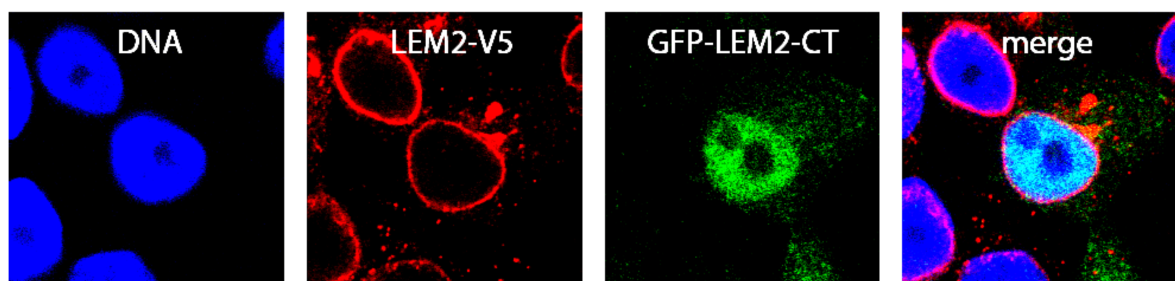


Figure 29: Immunofluorescence images of a stable HeLa cell line expressing full-length LEM2-V5 (red) and transiently expressing GFP-LEM2-CT (green).

GFP-LEM2-CT was found predominantly within the nucleus (Fig. 29), consistent with the proposed DNA-binding function of the MSC domain (Caputo *et al.* 2006). Localization of ectopic full-length LEM2 seemed unaffected by the co-expression of LEM2-CT (Fig. 29). However also GFP-LEM2-CT could not be stably expressed in cells, indicating a detrimental effect on viability probably by affecting chromatin functions.

LEM2 directly interacts with proteins of the nuclear periphery including A-and B-type Lamins, Emerin and MAN1

In collaboration with Malini Mansharamani and Kathy Wilson (JHU School of Medicine, Baltimore, USA) we investigated the binding properties of human LEM2 to the previously proposed binding partners, i.e. Lamins, Emerin and MAN1, in more detail. Consistent with our previous results, LEM2 was found to interact with A-type Lamins in a direct manner in blot overlay and microtiter assays (Fig. 30). Interestingly the affinity for disease-causing Lamin A mutants R527P and L530P, both causing Emery-Dreifuss Muscular Dystrophy, was reduced as compared to wild-type Lamin A or Lamin A mutants causing other forms of laminopathies, i.e. "R482Q" (causing Familial Partial Lipodystrophy) and "E578V" (causing Hutchison Gilford Progeria) (Fig. 30).

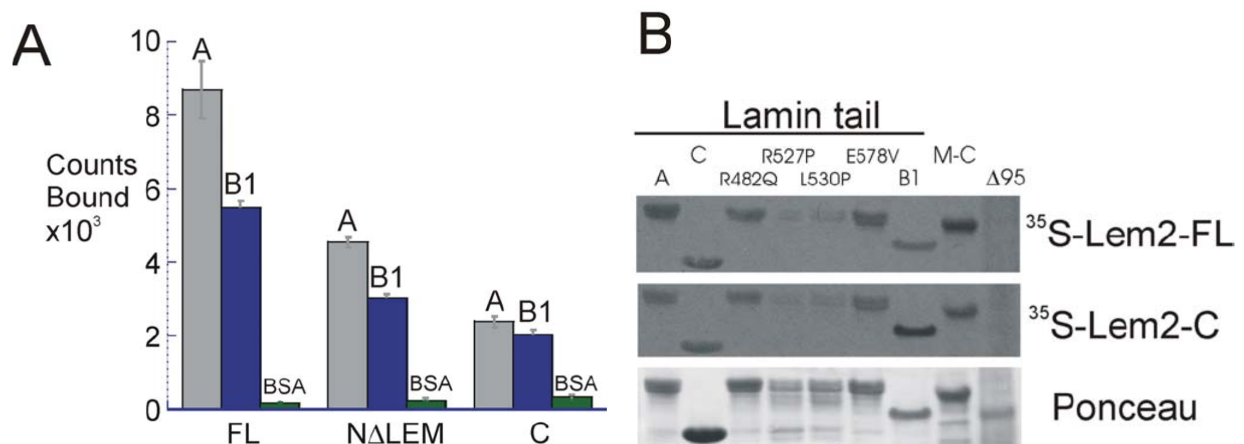


Figure 30: Direct binding of LEM2 fragments to lamins tested by microtiter and blot overlay assays. (A) Microtiter assay. Recombinant purified pre-Lamin A tail, Lamin B1 tail or purified BSA were immobilized in microtiter wells, incubated with equimolar amounts of ^{35}S-labeled LEM2-full length (FL), N-terminus of LEM2 ΔLEM (NΔLEM) or LEM2-C-terminus (C), then washed and counted (n=3 triplicate sets). Bars indicate standard deviations. (B) Blot overlay assays. Purified recombinant pre-Lamin A tail, Lamin C tail, pre-Lamin A tails containing single-point disease mutations R482Q, R527P, L530P, E578V, Lamin B1 tail, C-terminus of MAN1 (M-C) and Emerin mutant Δ95 (lacking residues 95-99; Δ95) were resolved in triplicate via SDS-PAGE: Two membranes were probed with either ^{35}S-LEM2 or ^{35}S-LEM2-C-terminus and autoradiographed (upper and middle panels), and a third gel was stained with Ponceau S (lower panel). Experiment was carried out by M. Mansharamani & K. Wilson (Baltimore, USA).

Additionally, these experiments revealed a second so far unknown Lamin-interacting region within the LEM2 C-terminus. Unexpectedly and contradicting our previous results, LEM2 bound Lamin B in these assays, though with about 30% reduced efficiency. Our previous experiments indicated an association of LEM2 with other LEM proteins of the INM, i.e. Emerin and MAN1

(Brachner *et al.* 2005). These interactions were confirmed in the *in vitro* binding experiments, revealing a direct interaction of LEM2 with MAN1 C-terminus (Fig. 29) and Emerin (Fig. 31).

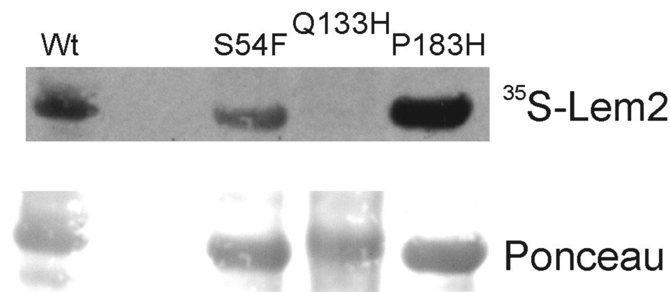


Figure 31: Crude lysates from bacteria expressing either wild type (WT) or disease mutant emerin proteins S54F, Q133H and P183H were resolved by SDS-PAGE, transferred to membrane and either probed with ^{35}S-LEM2-FL or stained with Ponceau to control for the amount of recombinant emerin protein in each lane. Experiment was carried out by M. Mansharamani & K. Wilson (Baltimore, USA).

Intriguingly LEM2 did not bind to the Emerin mutants "Δ95" and the disease causing mutation "Q133H" whereas disease-linked Emerin mutations "S54F" and "P183H" did not affect LEM2 binding (Fig. 30), clearly indicating a specific interaction between both proteins.

Summary and Conclusions

In this study, we aimed at investigating the role of LEM2's individual domains and the effects of ectopic expression of various LEM2 fragments on proposed LEM2 binding partners and on the cell cycle. Interestingly, we observed a subtle redistribution of Lamin A/C from the NE to the nucleoplasm upon expression of a LEM2 N-terminal fragment and an impairment of cell cycle progression. We speculate that these two effects are causally connected, because Lamin A/C has been linked to various cellular processes affecting proliferation. It is known for a long time that Lamin A/C is linked to differentiation but the molecular mechanisms remain unknown (Rober *et al.* 1989; Stewart and Burke 1987). Despite the putatively central function of Lamin A/C in differentiation, lmna deficient mice do not exhibit a severe deleterious phenotype until birth (Sullivan *et al.* 1999). Hence Lamin A/C is likely not essential for differentiation during development, but is probably involved in the establishment and maintenance of a fully differentiated phenotype in certain cell types during postnatal tissue homeostasis (Peter and Nigg 1991). Interestingly, the subnuclear distribution of Lamin A/C changes from a more nucleoplasmic to a mostly peripheral localization during differentiation of cells as described in myogenic and myelogenic *in vitro* differentiation models (Collard *et al.* 1992; Muralikrishna *et al.* 2001). LEM2 expression in muscle differentiation was also investigated in a study by Chen and colleagues, who reported that LEM2 (synonymously: NET25) is upregulated during C2C12

myoblast differentiation (Chen *et al.* 2006) – an effect which was never observed in our own experiments using a C2C12 clone in the lab (data not shown). In contrast, we noticed an inhibition of myoblast differentiation upon stable overexpression of LEM2 in C2C12 cells, probably pointing to an interference with cell cycle exit, consistent with the observed cell cycle phenotype of LEM2 overexpressing HeLa cells (described in this chapter).

It remains unclear, however, whether the increase of nucleoplasmic Lamin A/C triggered by overexpression of LEM2 N-terminus lead to cell death. Theoretically, this phenomenon could be caused by a checkpoint mechanism initiating cell cycle arrest, and finally apoptosis or necrosis. However, overexpression of LEM2-NT did not cause cell cycle exit as indicated by the positive Ki-67 staining and BrdU incorporation. Alternatively, competition of LEM2-NT with endogenous LEM2 at the NE and subsequent dislocation to the ER (as observed for ectopic full-length LEM2) could trigger cell cycle checkpoints and cause the observed effects.

In contrast to the effects observed upon expression of LEM2 fragments, we found higher proliferation rates in HeLa and C2C12 cells overexpressing full-length LEM2, and a mild shift of nucleoplasmic Lamin A/C to the periphery. In accordance with our results, Ulbert and colleagues have shown that knock-down of LEM2 impairs cell proliferation (Ulbert *et al.* 2006) and slightly delays cell cycle in G2 phase, although the authors did not comment whether cells additionally underwent apoptosis or necrosis.

Overall, the establishment of stable cell lines expressing LEM2 deletion constructs was inefficient, as compared to full-length LEM2 - most likely due to competition with endogenous LEM2 functions in nuclear architecture and cell cycle control. The observation that the lack of either the LEM or the MSC domain, thereby representing a membrane anchored LEM2 construct, negatively affected cell proliferation, while full-length LEM2 activated proliferation, pointed towards an important contribution of these domains to LEM2 function. The LEM domain could exert this function via binding BAF, which is involved in chromatin organization and gene expression (Margalit *et al.* 2007a). Intriguingly, BAF was recently reported to relocalize from the nucleoplasm to the cytoplasm in senescent or non-cycling cells, indicating a role of BAF in proliferation (Haraguchi *et al.* 2007). The MSC domain was found to be responsible for the formation of LEM2 patches at the NE, which recruit various resident proteins of the NE and the nuclear lamina together with chromatin. Thus, the impact on proliferation could be indirect by affecting other INM proteins, some of which are known to interfere with signaling pathways: MAN1 was shown to tether Smad proteins at the NE (Bengtsson 2007; Pan *et al.* 2005); Emerin was reported to regulate nuclear β-Catenin activity (Markiewicz *et al.* 2006).

The INM protein LBR (Lamin B-Receptor) has been shown previously to localize in microdomains along the NE, where it was suggested to form large assemblies of heterochromatin (Makatsori *et al.* 2004). We did not observe any overlap of LBR and LEM2 in immunofluorescence (Brachner *et al.* 2005). Hence, we propose Lamin A/C dependent complexes formed by LEM2 at the nuclear periphery including INM proteins, BAF and chromatin, which are clearly distinct from the microdomains formed by LBR.

Materials & Methods

Cloning strategy and plasmids

The eukaryotic expression vector pTB-hLEM2 has been described previously (Brachner *et al.* 2005). LEM2 fragments hLEM2-NT, hLEM2ΔLEM, hLEM2ΔMSC, hLEM2ΔLEMΔMSC were constructed by cloning PCR-generated sequences into pEntry-D-Topo vector (Invitrogen) via Topoisomerase reaction according to the recommendations of the manufacturer. Subsequently the fragments were shuttled into pDEST51 vector using Invitrogens' Gateway® technology as described before (Brachner *et al.* 2005). PCR primers used were:

hLEM2-139 forw 5'-CACCGCCATGGAGGAGCGGCTGCGGGAG-3' and hLEM2-SEX rev 5'-GCATCGCTCTGAGTCAGAGAAGGAAGAGG-3' to generate hLEM2ΔLEM, hLEM2-ATG forw 5'-CACCATGGCCGGCCTGTCGGACCTGGAACTGCGGC-3', hLEM2-1236 rev 5'-ATACATGGCTTGTTCCTCCTCTTC-3' for hLEM2ΔMSC as well as hLEM2-139 forw and hLEM2-1236 rev for hLEM2ΔLEMΔMSC.

LEM2 N-terminal fragment, LEM2-NT, was cloned accordingly: Primers hLEM2-ATG forw and hLEM2-585 rev 5'-AAGCTTCGCGCCAGCAGGGCCCGCTCGAGT-3' were used to generate the fragment, which was ligated into the pEntry-D-Topo vector. From the resulting pEntry-hLEM2-NT vector, the fragment was shuttled into either the pTracer or the pCDNA-myc plasmid (Invitrogen).

Cell culture, Transfection, Clonal Selection & Growth curves

HeLa cells were cultivated and transfected as described previously (Brachner *et al.* 2005). Clonal selection for stable expression of ectopic proteins was done after transfection by seeding of single cells and addition of the antibiotic Blasticidin (Invitrogen) to the growth medium (20µg/mL) for two weeks. Single colonies were picked using cloning cylinders and expanded for further analysis. Growth curves were determined by seeding of 10^5 cells to petridishes (6 cm diameter) at day 0 and counting of total cell numbers in one petridish each day. Cell counting was done with a "Casy Cell Counter" (Casy). Curves were plotted using "Excel" (Microsoft).

Immunofluorescence microscopy & BrdU incorporation assay

Fixation and staining procedures were done as described previously (Brachner *et al.* 2005). Antibodies used for staining were mouse and rabbit anti-V5 (Invitrogen and Sigma, respectively), a BAF antiserum was kindly provided by K. Furukawa (Furukawa 1999), sera against LAP2α and LAP2β have been described previously (Dechat *et al.* 1998; Vlcek *et al.* 2002). Anti-Lamin A (clone 133A2) was purchased from Abcam, the monoclonal antibody against Lamin A/C was generously provided by F. McKeon (Loewinger and McKeon 1988) and the mouse anti-Lamin B1 antibody (8D1) was a kind gift of D. Vaux (Maske *et al.* 2003).

BrdU labeling was performed using the "BrdU labeling and detection kit I" (Roche), essentially according to the manufacturers manual. Briefly, cells were seeded on cover slips the day before labeling at various densities, transfected with Lipofectamine 2000 (Invitrogen) following the manufacturers instructions and BrdU labeled for 30 min the following day. After the incubation time, cells were fixed with ice-cold EtOH/50mM Glycine pH2.3 at -20°C for 20 min, followed by standard immunofluorescence staining procedures. Cells positive for BrdU incorporation were counted and numbers were compared to untransfected cells in the same culture and to untreated control cells.

3.2.3 Yeast *SRC1* (*HeH1*) and Human LEM2 are Orthologous Proteins with Functional Conservation Throughout Evolution

The nucleus represents the most prominent feature of eukaryotic cells and is separated from the cells' cytoplasm by a lipid bilayer, the nuclear envelope (see also chapters 2.1 and 2.2). Despite the obvious structural similarities of the nuclear envelope (NE) and nuclear pore complexes (NPCs) in unicellular organisms, such as *S.cerevisiae*, and in higher eukaryotes, little is known about functional conservation of specific constituents of the NE.

The starting point of this study was a computational analysis of genomic databases aiming at the identification of genes orthologous to human LEM2 in other species. Surprisingly we retrieved a putative ortholog in *S.cerevisiae* with significant similarities in primary sequence and in domain topology. Consequently, we intended to investigate the phylogenetic relationship of the mammalian inner nuclear membrane protein LEM2 and its proposed orthologous protein in yeast, *SRC1*, on a functional level, exploiting a collaboration with Susana Rodriguez-Navarro (Centro de Investigación Príncipe Felipe, Valencia, Spain), who has described a *SRC1*$^{-/-}$ yeast strain previously (Rodriguez-Navarro *et al.* 2002).

Results

Comprehensive searches for LEM2-related sequences in genomic databases revealed the yeast gene *SRC1* (or *HeH1* according to a different nomenclature) (King *et al.* 2006) as a putative orthologous protein to human LEM2 or MAN1 (see also chapter 3.1). Besides *S.cerevisiae*, also several other unicellular organisms contain this conserved gene, suggesting a strong functional conservation of the protein throughout evolution. All so far available sequences share a common domain topology including a N-terminal SAP or LEM motif, two transmembrane domains as well as the highly conserved C-terminal MSC domain (Fig. 32). In a recent study, King and colleagues (King *et al.* 2006) concluded that the yeast proteins *HeH1* (synonymous to *SRC1*) and *HeH2* are orthologous to mammalian LEM2 and MAN1. Furthermore, a detailed computational study performed by Mans and colleagues also proposed that MAN1 and *SRC1* proteins are evolutionary conserved INM proteins (Mans *et al.* 2004).

In order to analyze the evolutionary relationship of the SRC1 family and the LEM2/MAN1 family we performed comprehensive sequence alignments and calculated the similarities of the two important motifs within these proteins, the SAP/LEM and the MSC domain. As the major difference between SAP and LEM domain is the direct versus indirect way to interact with chromatin we included the LEM-like motif of human LAP2, representing a related, proposed direct DNA binding motif (see also chapter 3.1) in the analysis.

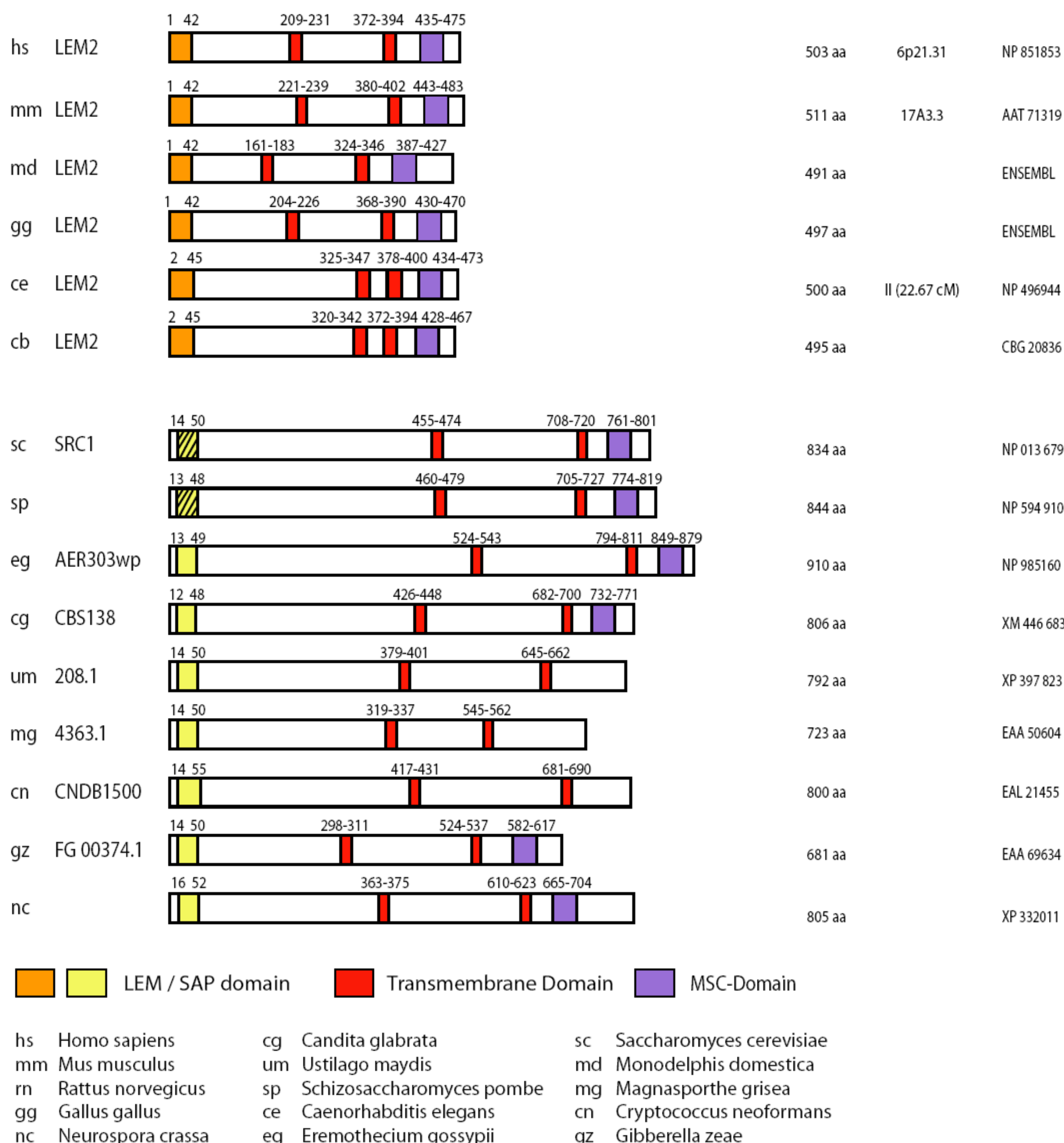
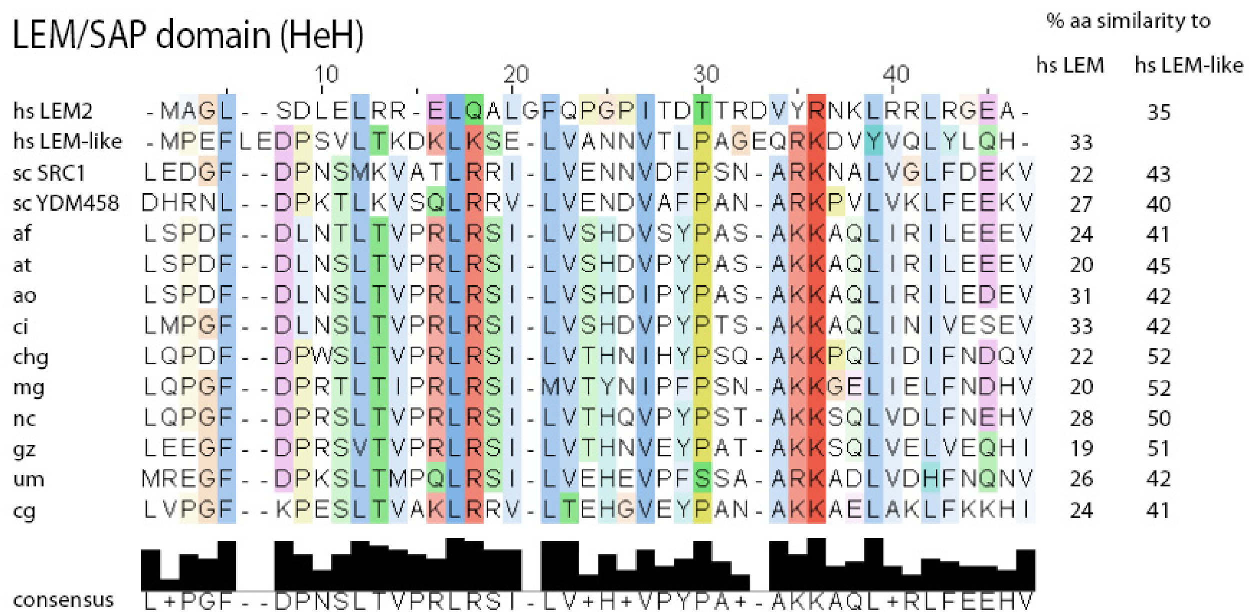


Figure 32: Predicted domain topology of metazoan LEM2 proteins and of orthologous proteins in unicellular eukaryotes.

Interestingly, all analyzed SAP motifs, which are thought to resemble a helix-extension-helix structure (therefore sometimes designated as “HeH” motif) are significantly more closely related to the LEM-like motif than to the canonical BAF-interacting LEM domain. Whereas the similarity between the LEM domain and SAP-like motifs did not exceed approximately 30% at primary sequence level, the conservation to the LEM-like motif was up to 52% (Fig. 33). The C-terminal MSC motif, reported to form a winged-helix DNA binding motif (Caputo *et al.* 2006), was found to be well conserved among human LEM2/MAN1 and its orthologous proteins in protozoan organisms, accomplishing about 40% of similarity at amino acid level (Fig. 33).



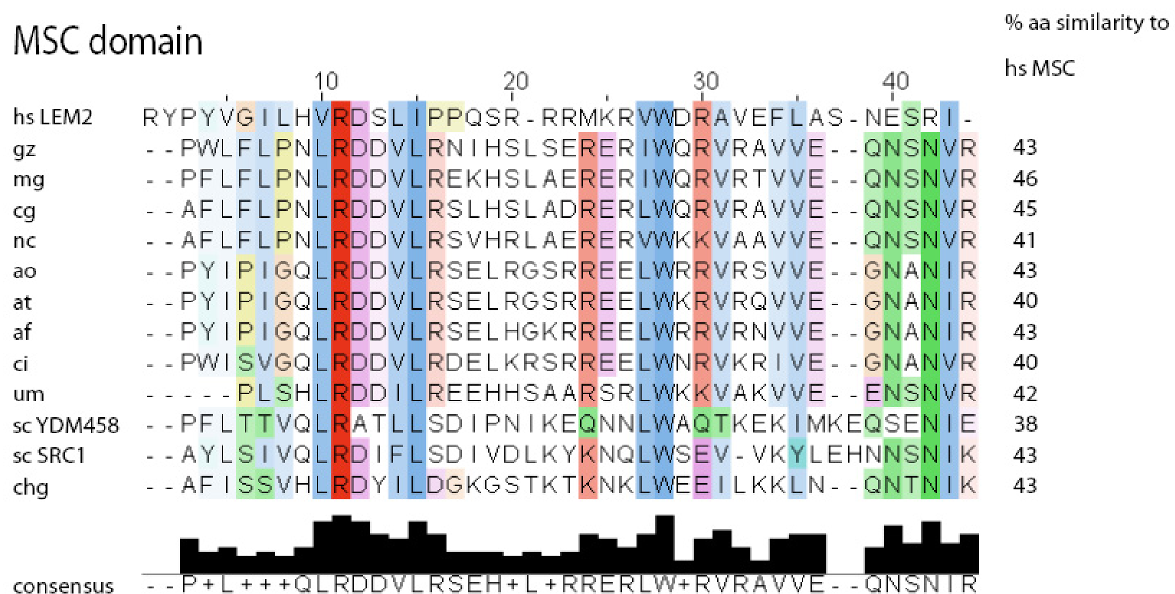


Figure 33: Conservation of the LEM/SAP domain and the C-terminal MSC domain among the SRC1 protein family. Abbreviations of species as indicated in Figure 31.

Therefore, we speculate that the evolutionary conserved LEM2/MAN1/*SRC1* protein organization may have essential functions, probably in organizing peripheral chromatin.

Starting from a mild phenotype in sister chromatid segregation in src1$^{-/-}$ yeast cells, Rodriguez-Navarro and colleagues tested for synthetic interactions between various mutants known to be involved in sister chromatid segregation and *SRC1*. While no additional phenotype was observed in case of *cdc20-/-src1-/-* (*Cdc20* ubiquitinates *Securin* to liberate *Separin* which then is able to cleave *Cohesin*. After *Cohesin* cleavage, sister chromatid segregation is initiated.), both *esp-/-* and *scc-/-* (encoding yeast *Separin* and *Cohesin* respectively) failed to grow without *SRC1*,

thereby clearly suggesting a role of *SRC1* in chromatid segregation (Rodriguez-Navarro *et al.* 2002). In order to confirm the existence of an evolutionary conserved molecular function of LEM2 and *SRC1* on a genetic level directly, human LEM2 was tested for functional complementation capacity in the temperature-sensitive *esp*$^{-/-}$*src1*$^{-/-}$ yeast strain.

Ectopic overexpression of human LEM2 and MAN1 in yeast cells was verified by Western blot analysis on protein level (Fig. 34A) and intracellular localization was tested by fluorescence microscopy in living cells (Fig. 34B). We concluded from these initial experiments that ectopic expression of LEM2 is not detrimental to yeast cells, as strong expression levels of the LEM2-V5 construct were achieved in yeast. MAN1 was expressed only at low levels, probably due to toxicity (Fig. 34A). Intriguingly, fluorescence microscopy revealed that human LEM2-RFP is recruited to the yeast NE, although the nuclei of unicellular eukaryotes contain no nuclear lamina (see also chapter 2.3). Whether the LEM2-RFP construct localizes at the inner or outer nuclear membrane could not be determined. Recently King and colleagues proposed an NLS in INM membrane proteins targeting these proteins to the INM (King *et al.* 2006). As two predicted NLS were also present in human LEM2, they may mediate the targeting of LEM2-RFP to the yeast INM. The localization of hLEM2 at the yeast NE could be further stabilized by the MSC domain, which may interact with peripheral chromatin. The LEM domain is supposed to be inactive in yeast due to the lack of a BAF ortholog in yeast (Margalit *et al.* 2007a), but the existence of so-far unknown interaction partners can not be excluded.

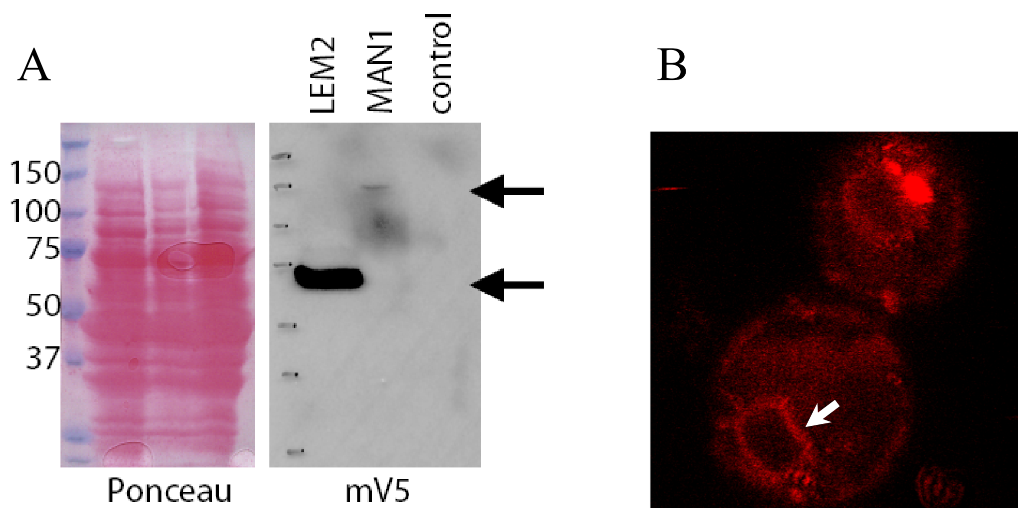


Figure 34: (A) Human LEM2 and MAN1 ectopically expressed in yeast. Left image: Ponceau-stained Western blot of yeast extract, right image: the same blot probed with anti-V5 antibody. Control: untransformed cells. Arrows: Expressed V5-tagged proteins at correct heights. (B) Confocal fluorescence image of living yeast cells transformed with hLEM2-RFP. Arrow: Yeast NE.

The results of the complementation assays showed that both, *SRC1* and human LEM2 were able to complement the growth phenotype as compared to empty vector controls (Fig. 35A). This clearly indicates the postulated phylogenetic relationship of mammalian LEM2 proteins and yeast *SRC1*, not only at the level of sequence conservation, but also functionally. To further elaborate the domains of LEM2 involved in complementation, we tested two LEM2 deletion constructs. Intriguingly, the MSC domain of LEM2 as well as the insertion of the construct into the membrane was found to be essential for complementation, as the constructs hLEM2ΔMSC and hLEM2ΔTM failed to complement the $esp^{-/-}src1^{-/-}$ growth phenotype. The unrelated human NE protein Nurim and an empty control vector were used as negative controls (Fig. 35A). Additionally, we tested for complementation ability of human MAN1 (Fig. 35B), but the results are still unclear as the complementation was much weaker compared to hLEM2 and the expression level of MAN1 in yeast remained very low for unknown reasons.

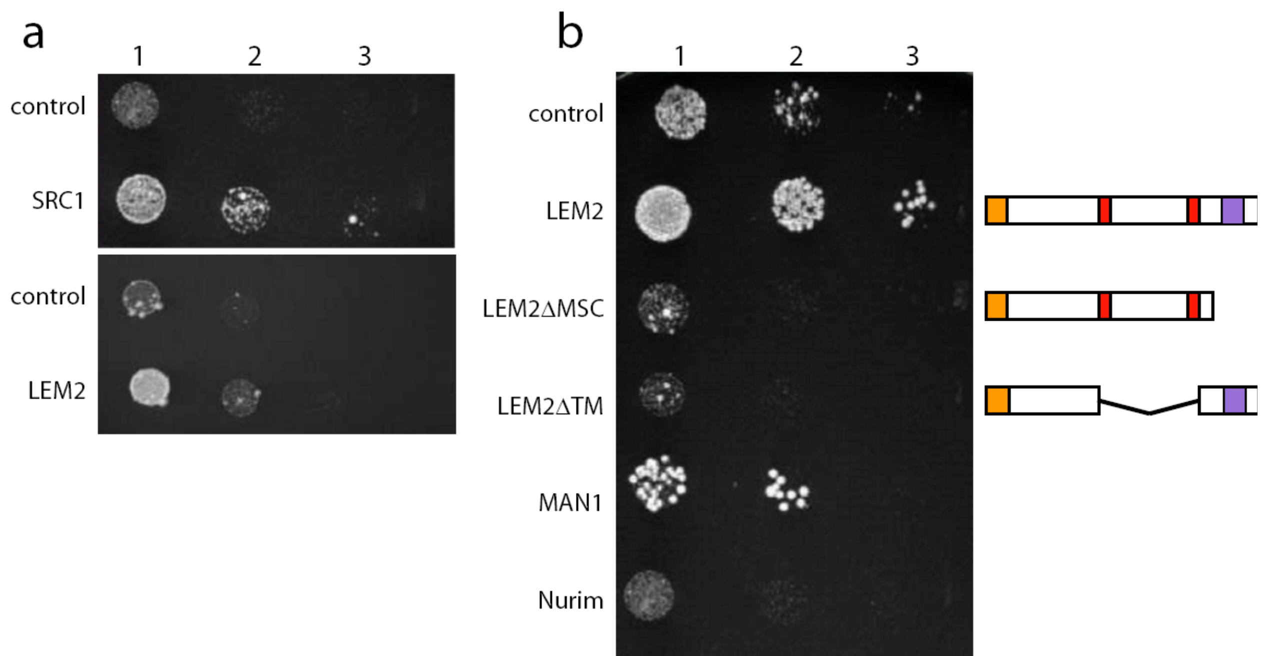


Figure 35: Complementation assay in a $esp^{-/-}src1^{-/-}$ temperature sensitive yeast strain. Lanes 1: Growth at permissive temperature. Lanes 2 and 3: Growth at restrictive temperature, two different time points. (a) Yeast transformed with empty control vector (control) or expression vectors containing SRC1 or human LEM2. (b) Yeast transformed either with empty control vector (control) or with expression vectors containing either human LEM2 constructs (as depicted at the right hand side), human MAN1 or Nurim. Right hand side: Schematic LEM2 constructs: Orange rectangle: LEM domain, red: transmembrane domain, violet: MSC motif.

Nonetheless our data show for the first time the functional conservation of a yeast NE protein throughout evolution, and we could narrow down one functionally relevant region to a specific domain present in *SRC1* and LEM2, representing a C-terminal DNA binding motif (Caputo *et al.* 2006). These conserved functions of the proteins may include roles in NE integrity, in organization of chromatin at the nuclear periphery, or in cell cycle control (see also previous chapters of this thesis). Also a function in sister chromatid segregation might be envisaged, considering the genetic link to *src1* mutants and our observation of linked nuclei of adjacent hLEM2-overexpressing cells after mitosis.

Summary & Conclusions

LEM2/MAN1 and *SRC1*-like proteins form an evolutionary conserved family of INM proteins, as it was suggested in several reports before (Brachner *et al.* 2005; King *et al.* 2006; Mans *et al.* 2004). These conclusions were solely on computational prediction. We have shown the phylogenetic relationship *in vivo* by genetic means directly.

The functional conservation of LEM2 throughout evolution is proposed by several findings.

(1) Ectopic human LEM2 was targeted to the NE in yeast cells. (2) Complementation assays in a *esp*$^{-/-}$*src1*$^{-/-}$ yeast background revealed that human LEM2 partially compensates the growth phenotype caused by the lack of yeast *SRC1*. Based on the analysis of various LEM2 deletion constructs we propose an essential role of the interaction of the MSC motif with DNA. It remains unclear whether LEM2/*SRC1* fulfils specific roles in the structural organization of the nuclear periphery in the interphase nucleus or whether it is involved in mitosis as suggested by data obtained from *src1*$^{-/-}$ cells (Rodriguez-Navarro *et al.* 2002). Interestingly, proteomic screens employing the yeast-two-hybrid technology, identified *SRC1* to interact with a component of the GINS-complex, which might be involved in DNA replication (MIPS database, http://mips.gsf.de/genre/proj/yeast/) (Ito *et al.* 2001; Labib and Gambus 2007) and with several other nuclear components, including *MCD1*, a protein involved in sister chromatid cohesion, the cyclin dependent kinase *CDC28* and components of the chromatin remodeling

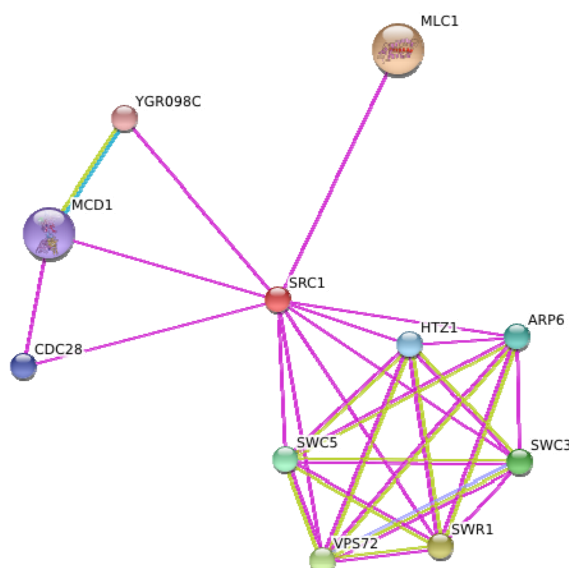


Figure 36: Summary of proposed SRC1 interactions. Adapted from the "STRING" database (http://string.embl.de/).

complex *SWR1* (Fig. 36). The interaction with chromatin via N-and C-terminal domains may be a common, evolutionary conserved property of all LEM2/MAN1 and *SRC1*-like proteins, although the molecular function of this “forceps”-type binding to DNA has still to be determined. The “replacement” of the N-terminal SAP motif by a LEM domain during evolution might reflect the evolutionary force to increase the complexity of regulation of the chromatin-NE interactions in metazoan organisms. This was achieved by introducing a “novel” mediator protein that regulates the binding of the LEM domain to chromatin. Noteworthy, a recent study performed by Hattier and colleagues revealed interesting findings upon overexpression of *HeH1* and *HeH2* in yeast. The authors demonstrated that excess of *HeH1* and *HeH2* perturbs the yeast NE, is highly toxic to cells and distorts the chromatin organization, thereby corroborating a proposed function of both proteins in NE structure and chromatin organization (Hattier *et al.* 2007).

In *C.elegans*, knock-down of ce-LEM2 or ce-Emerin alone had no dramatic effect on cell viability (about 15% dead embryos), whereas the knock-down of both membrane anchored LEM proteins was detrimental for almost 100% of embryos (Liu *et al.* 2003), strongly suggesting redundant functions of these INM proteins. *Saccharomyces cerevisiae* contains a second related gene, encoding a currently undescribed protein, termed *HeH2* (or *YDR458*) (King *et al.* 2006; Rodriguez-Navarro *et al.* 2002), as well as a shorter splice variant of *HeH1*, which lacks the C-terminal transmembrane domain and the MSC motif (Fig. 37).

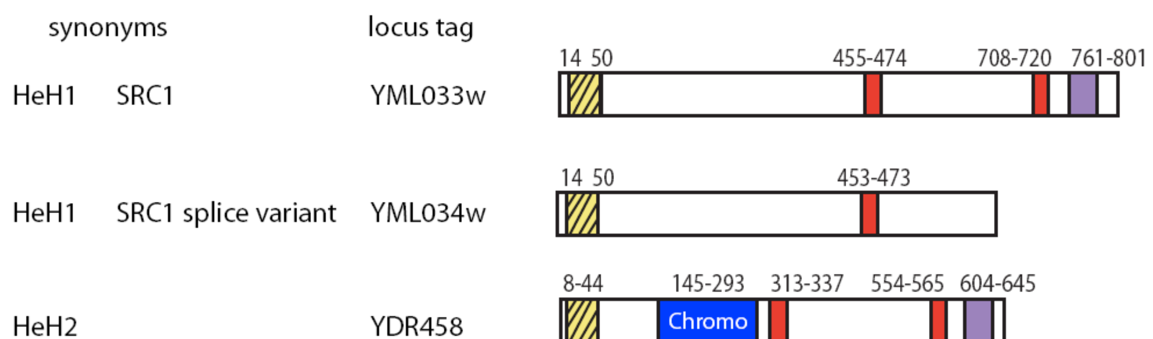


Figure 37: Predicted domains in yeast Helix-extension-Helix proteins 1 and 2. Yellow rectangle: SAP-like motif, blue box: Chromo-Domain, red: transmembrane domains, violet: MSC domain.

In analogy to *C.elegans*, *HeH1* (*SRC1*) and *HeH2*, may have redundant functions in yeast, although no evidence for a functional redundancy was reported so far by Rodriguez-Navarro and colleagues (Rodriguez-Navarro *et al.* 2002). Nevertheless, it would be very exciting to investigate *heh1*$^{-/-}$*heh2*$^{-/-}$ double knockout cells and to combine *heh1* or *heh2* deficiency with *esp* or *scc* knockouts. Currently these experiments are planned by our collaborators and will probably provide deeper insights into the functions of the LEM2/*SRC1* superfamily.

Materials & Methods

Cloning strategy and plasmids

The yeast expression vectors containing hLEM2, hLEM2ΔMSC, hLEM2ΔTM, hMAN1 were cloned by digesting the mammalian expression vectors “JG132” (full length hLEM2), “AB82” (hLEM2ΔMSC), “JG187” (hLEM2ΔTM) and “AB46” (hMAN1) with restriction enzymes SpeI and PmeI, thereby retrieving fragments containing the respective inserts fused to the 6xHis and V5-tag of the pTracer vector. Fragments were gel eluted and ligated into the SpeI/SmaI sites of the yeast expression vector p416-GPD plasmid. Human Nurim was amplified by PCR from a human mixed tissue cDNA sample.

Primers used were hNurim-forw 5’-CAGGAATTCATGGCCCCTGCACTGCTC-3’ and hNurim-rev 5’-TATGTCGACTCACTCTGCCTCCCCATCC-3’, the fragment was digested with EcoRI and SalI, and ligated into the corresponding sites of p416-GPD. The RFP-tagged hLEM2 was cloned by inserting the PCR generated and SpeI/XhoI cut monomeric RFP (Long *et al.* 2005) into the XbaI and SalI sites of p416-hLEM2.

Primers used were mRFP-forw 5’-GTCGACTAGTCATGACTGGTGGACAGCAAATG-3’ and mRFP-rev 5’-GTATCTCGAGTTAGGCGCCGGTGGAGTG-3’)

Computational Analysis

In-silico analyses of sequences as well as computational prediction of protein motifs were performed essentially as described previously (Brachner *et al.* 2005). Sequence alignments and calculation of consensus sequences were done with the ClustalW software (http://www.ebi.ac.uk/Tools/clustalw).

Live-cell fluorescence microscopy

Yeast cells were transformed with the p416-hLEM2-RFP construct and selected for uracil independent growth. For fluorescence microscopy living cells were distributed in ibidi-treat® slides (ibidi) and imaged on a LSM-Meta confocal laser scanning microscope (Zeiss). Images were adapted for brightness and contrast using the Zeiss LSM Image Browser (Zeiss) and mounted with programs Photoshop and Illustrator (Adobe).

Immunoblotting

Cell lysis, PAGE and immunoblotting were performed as described previously (Brachner *et al.* 2005).

3.2.4 LEM2, Conclusions & Outlook

Biochemical analyses revealed that LEM2 is a novel LEM protein at the INM which is associated with A-type Lamins (Brachner *et al.* 2005). Subsequent studies reported (1) that LEM2 is upregulated during myodifferentiation (Chen *et al.* 2006), (2) that LEM2 is essential for the maintenance of the nuclear shape in human cells (Ulbert *et al.* 2006) and (3) that LEM2 represents an evolutionary conserved gene with orthologs in unicellular organisms (Brachner *et al.* 2005; King *et al.* 2006; Mans *et al.* 2004) (chapter 3.1). Recently, we could show that LEM2 was conserved throughout evolution not only by sequence and domain topology of but also by localization at the NE and its particular functions (chapter 3.2.3). Conserved LEM2 functions may include the structural organization of the NE, chromatin organization at the nuclear periphery, as suggested by proposed *HeH1* (*SRC1*) binding partners and observations upon overexpression of *HeH1* and *HeH2* ((Hattier *et al.* 2007) and chapter 3.2.3), and a role in sister chromatid segregation, based on the reported phenotype of *src1*$^{-/-}$ yeast cells (Rodriguez-Navarro *et al.* 2002). Intriguingly, human LEM2ΔMSC did not rescue the growth phenotype of *esp*$^{-/-}$*src1*$^{-/-}$ deficient yeast (chapter 3.2.3) pointing to a direct link between LEM2 functions and MSC domain indicated chromatin organization.

MAN1, another LEM domain protein at the INM, is closely related to LEM2 regarding sequence and domain topology. Both proteins bind directly to A-type Lamins, BAF and Emerin (Brachner *et al.* 2005; Mansharamani and Wilson 2005), and we could show that they also interact with each other *in vitro* (chapter 3.2.2). However, also non-redundant functions of both proteins were described. The most characteristic property specific for MAN1 is the direct binding to Smads via its unique C-terminal "RRM" motif, which is missing in LEM2 proteins (Brachner *et al.* 2005; Pan *et al.* 2005). This finding raises the question whether LEM2 might act as a negative regulator of MAN1 functions by competitive binding to Lamin A/C, Emerin and BAF.

Overexpression of full-length LEM2 in cells revealed an interesting role of the C-terminal winged-helix type DNA binding motif identified originally by Caputo and colleagues in MAN1 (Caputo *et al.* 2006), and termed MSC domain in LEM2 (Brachner *et al.* 2005; Mans *et al.* 2004). The analyses of various deletion constructs showed that the MSC motif is essential for the formation of LEM2 patches at the NE (chapter 3.2.2). Holaska and colleagues purified several distinct Emerin-containing NE complexes from HeLa cells, proposing that each complex is involved in a specific nuclear process (Holaska and Wilson 2007). Schirmer and Gerace suggested that INM proteins form tissue specific complexes with other INM proteins, transcription factors and chromatin at the INM. They argue that these platforms are essential to establish and maintain the differentiated state of specific cells (Schirmer and Gerace 2005).

Interestingly, expression of N-terminal LEM2 fragments, which contain the Lamin A/C-interacting region of LEM2 lead to a subtle relocalization of peripheral Lamin A/C to the nucleoplasm. Expression of these constructs in cells inhibited cell cycle progression but did not induce quiescence or senescence (chapter 3.2.2). A cell cycle defect was reported also upon RNAi-mediated down regulation of LEM2 in cells (Ulbert *et al.* 2006). A possible explanation for these findings may be that a lack of LEM2 at the INM or the overexpression of LEM2 N-terminal fragment triggers a checkpoint mechanism mediated by an excess of nucleoplasmic Lamin A/C.

Although a striking cellular phenotype was reported upon downregulation of LEM2 in cell lines (Ulbert *et al.* 2006), it seems problematic to me to engineer a LEM2-deficient mouse model as its functions might be compensated by other INM proteins, especially by MAN1. Functional compensation was observed in a *emerin*$^{-/-}$ mouse (Lammerding *et al.* 2005) as well as in *lem-2* deficient *C.elegans* (Liu *et al.* 2003).

LEM proteins have multiple functions in cell structure, chromatin & signaling

Metazoan organisms, including unicellular species, contain complex, intricate networks of molecular switches, signal transducers and building blocks, in which resident proteins often have (1) a multitude of interaction partners and (2) often fulfill more than a one function. Not surprisingly, both, a plethora of interacting molecules and several (partially redundant) functions were found for the nuclear Lamins and the LEM protein family. The nuclear lamina and LEM proteins at the INM have essential functions in the maintenance of nuclear shape and nuclear positioning within the cell, exert essential roles in chromatin organization at the nuclear periphery and modulate specific signaling pathways. Accordingly, various and often mosaic phenotypes are observed in envelopathy patients bearing mutated versions of these proteins. Hence, the pathological phenotype reflects the role of these proteins in nuclear structure, chromatin organization *and* signaling.

LEM proteins and INM proteins may function as integration platform, connecting the cytoplasmic cytoskeleton with a proposed nucleoskeleton. They may also recruit transcription factors and signaling molecules and tether chromatin to the nuclear envelope. Therefore, the nuclear periphery represents not only a structural enforcement between cytoplasm and nucleus, but provides an integrative interface to convert and execute signals from in-and outside the nucleus. The family of LEM proteins exhibit many features allowing them to fulfill such a multitude of integrative functions: INM localization, binding partners (as identified so far), ability to interact with chromatin and potential involvement in signaling pathways.

3.3 LEM3

The LEM3 sequence has been annotated in several computational screens aiming to identify novel members of the LEM-domain containing-, Ankyrin repeat containing-, or GIY-YIG protein family (chapter 3.3.1). In this study we identified LEM3 as a novel, highly conserved LEM protein, present in all investigated species from *C.elegans* to man.

Analyses of the expression pattern revealed that LEM3 is transcribed in a tissue-restricted manner, indicating a function in lymphoid cells. Furthermore, the LEM3 sequence was found to be subjected to alternative splicing, yielding three different isoforms. Intriguingly, two isoforms lack half of the LEM domain as well as varying parts of the predicted C-terminal GIY-YIG motif, suggesting regulatory functions of the full-length protein.

Ectopic expression of GFP-or V5-tagged human LEM3 revealed a cytoplasmic localization and a significant association with Actin filaments, as well as γ-Tubulin. Artificial inhibition of nuclear export by administration of Leptomycin B leads to a strong accumulation of LEM3 in the nucleus, indicating nucleo-cytoplasmic shuttling of LEM3. In contrast to human LEM3, murine LEM3 localized within both, cytoplasm and nucleus in untreated cells, but like hLEM3, mLEM3 co-localized with Actin filaments and γ-Tubulin in the cytoplasm. Additionally, we found that nuclear LEM3 accumulated in speckles, which co-localized with splicing factor SC-35 and U snRNPs, suggesting a role in processing nucleic acids.

The prediction of a GIY-YIG motif, a known structural domain involved in DNA cleavage and recombination, in LEM3 lead us to speculate about a possible function of LEM3 in DNA damage response. Therefore, we performed DNA damage experiments to elucidate any potential involvement of LEM3 in this particular cellular function. Surprisingly, we found that expression of LEM3 lead to a strong phosphorylation of DNA damage specific Histone H2A.X, irrespective of induced DNA damage. This effect was even more pronounced when human LEM3 was forced to accumulate in the nucleus by Leptomycin treatment. It remains unknown whether DNA damage and Histone H2A.X phosphorylation are direct consequence of LEM3 overexpression, probably due to the function of the GIY-YIG motif, or caused by an indirect mechanism, such as induction of apoptosis.

3.3.1 LEM3 (ANKRD41) is a Novel LEM-Domain Protein that Shuttles between Nucleus and Cytoplasm and Affects BAF

LEM3 (ANKRD41) is a novel LEM-domain protein that shuttles between nucleus and cytoplasm and affects Barrier-to-Autointegration Factor

Andreas Brachner[†], Ayelet Margalit*, Rachel Barkan*, Yosef Gruenbaum*, Roland Foisner[†#] and Josef Gotzmann[†#]

Max F. Perutz Laboratories, University Departments at the Vienna Biocenter, [†]Department of Medical Biochemistry, Medical University of Vienna, and *Department of Genetics, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel.

Short title: Nucleo-cytoplasmic shuttling of LEM3

Keywords: Barrier-to Autointegration factor, lamina, LEM-domain, nuclear transport,

[#] Authors for correspondence:

Roland Foisner
Max F. Perutz Laboratories
Medical University Vienna
Dr. Bohr-Gasse 9
A-1030 Vienna
roland.foisner@meduniwien.ac.at

Josef Gotzmann
Max F. Perutz Laboratories
Medical University Vienna
Dr. Bohr-Gasse 9
A-1030 Vienna
josef.gotzmann@meduniwien.ac.at

Abstract

The LEM domain is a 40 residues long structural motif that binds the chromatin-associated Barrier-to-Autointegration factor (BAF). Most of the characterized LEM-domain proteins reside in the inner nuclear membrane and bind lamins. Here we investigate a novel evolutionarily conserved LEM-domain protein lacking a transmembrane domain termed LEM3. Mammalian LEM3 encodes three alternatively spliced isoforms. The two smaller isoforms are missing part of the LEM-domain. Only the longest LEM3 isoform containing the complete LEM domain can bind BAF. Human LEM3 is mostly cytoplasmic and associates with actin stress fibers, while murine LEM3 localizes primarily to the nucleoplasm. Inhibition of nuclear export caused the accumulation of ectopically expressed human LEM3 in the nucleus and the mislocalization of BAF, suggesting that human LEM3 shuttles between nucleus and cytoplasm and interacts with BAF. RNA interference mediated knockdown in *C. elegans* showed that LEM-3 is not an essential gene and is not functionally redundant with the other *C. elegans* LEM proteins, LEM-2 and Emerin.

Introduction

The nuclear lamina is composed of lamins and lamin-associated proteins. It is positioned underneath the inner nuclear membrane (INM) and is involved in the dynamic organization of the nuclear envelope and peripheral chromatin (Foisner, 2001; Gruenbaum et al., 2005; Margalit et al., 2005b; Prunuske and Ullman, 2006; Schirmer and Foisner, 2007; Vlcek and Foisner, 2007). Several INM proteins share an N-terminal-located structural motif of 40 amino acids length, termed the LEM domain (Laguri et al., 2001). LEM domain proteins have important and essential functions in regulating nuclear architecture and mitosis, and in cell signaling and gene expression (Margalit et al., 2005b; Wagner and Krohne, 2007). Mutations in human LEM proteins emerin, LAP2 and MAN1 cause human diseases affecting striated muscle and bone (Hellemans et al., 2004).

One shared feature of all characterized LEM domain proteins is an interaction with a sequence-independent DNA-binding and DNA-cross-linking molecule, termed Barrier-to-Autointegration Factor (BAF) (Cai et al., 2001; Margalit et al., 2007a; Shumaker et al., 2001), thereby establishing dynamic protein-chromatin interactions at the nuclear periphery. LEM protein - BAF complexes are involved in nuclear envelope assembly and chromatin re-organization after mitosis in *C. elegans* (Liu et al., 2003; Margalit et al., 2005a) and in mammalian cells (Dechat et al., 2004; Haraguchi et al., 2001; Shimi et al., 2004). Furthermore, LEM protein – BAF complexes were implicated in viral DNA integration into the host genome and in the regulation of gene expression (Margalit et al., 2007a).

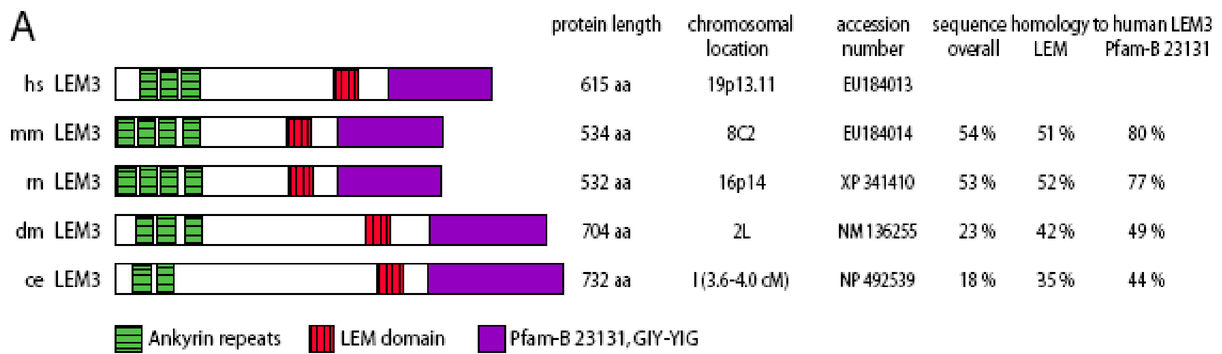
Apart from the well characterized LAP2, emerin and MAN1 other LEM genes have been identified and partially characterized: LEM2, a MAN1-related protein in mammalian cells (Brachner et al., 2005; Chen et al., 2006; Ulbert et al., 2006) and Otefin and Bocksbeutel in *Drosophila* (Padan et al., 1990; Wagner et al., 2004). The availability of the entire genome sequence of an increasing number of organisms allowed the *in silico* identification of several novel, so far uncharacterized putative LEM protein family members. In mammals these include LEM-domain-containing 1 (LEM5) (Lee and Wilson, 2004; Yuki et al., 2004), LEM3 and LEM4 (Lee et al., 2000; Lee and Wilson, 2004).

In this study we describe the initial biochemical and cell biological characterization of LEM3, which is the only so-far known LEM protein that is conserved from *C. elegans* to man. LEM3 contains ankyrin repeats and has a centrally located LEM domain, but lacks a transmembrane domain. It shuttles between the cytoplasm and nucleus, binds BAF and affects BAF localization when overexpressed in cells.

RESULTS

LEM3 is highly conserved in metazoans

The *LEM3* gene has been annotated previously in computational screens for novel protein family members characterized by the presence of a LEM-domain, ankyrin-repeats, or a GIY-YIG-motif (Dunin-Horkawicz et al., 2006; Lee et al., 2000; Lee and Wilson, 2004). All assembled LEM3 sequences in the ENSEMBL database (http://www.ensembl.org /index.html), which are also annotated as ANKRD41 (Ankyrin repeat domain protein #41), reveal a putative LEM domain (Fig. 1A, red box) in the middle of the polypeptide (aa 342-385 in humans), 2-4 ankyrin repeats at the N-terminal domain depending on the species (green box, 3 repeats between aa 25-125 in hLEM3), and a putative C-terminal GIY-YIG motif (violet box, aa 431-601 in humans), which has also been described as B23131 in the Pfam-B database (http://www.sanger.ac.uk/Software/Pfam/) as a nucleases-specific domain potentially involved in DNA recombination and/or DNA repair (Dunin-Horkawicz et al., 2006). Human LEM3 (hLEM3) contains 615 residues with a predicted molecular mass of 65 kD and a pI of 6.0, while murine LEM3 (mLEM3) is considerably smaller, comprising 534 amino acids with a predicted molecular mass of 56 kD and a pI of 6.2. Overall, primary sequence homologies between mammalian LEM3 proteins are around 54% with the C-terminal GIG-YIG domain showing the highest homology (~80%), while LEM3 orthologs in nematodes and arthropodes were 18% and 23% identical to hLEM3 at primary sequence level, respectively (Fig. 1A). The molecular domain organization of LEM3 orthologs is conserved in representative organisms of major metazoan clades, including Vertebrata, Arthropoda, Tunicata, Nematoda and Echinodermata (Fig. 1B). This conservation has not been observed for other LEM proteins including Emerin, LAP2, MAN1, LEM2 and LEM5 (data not shown).



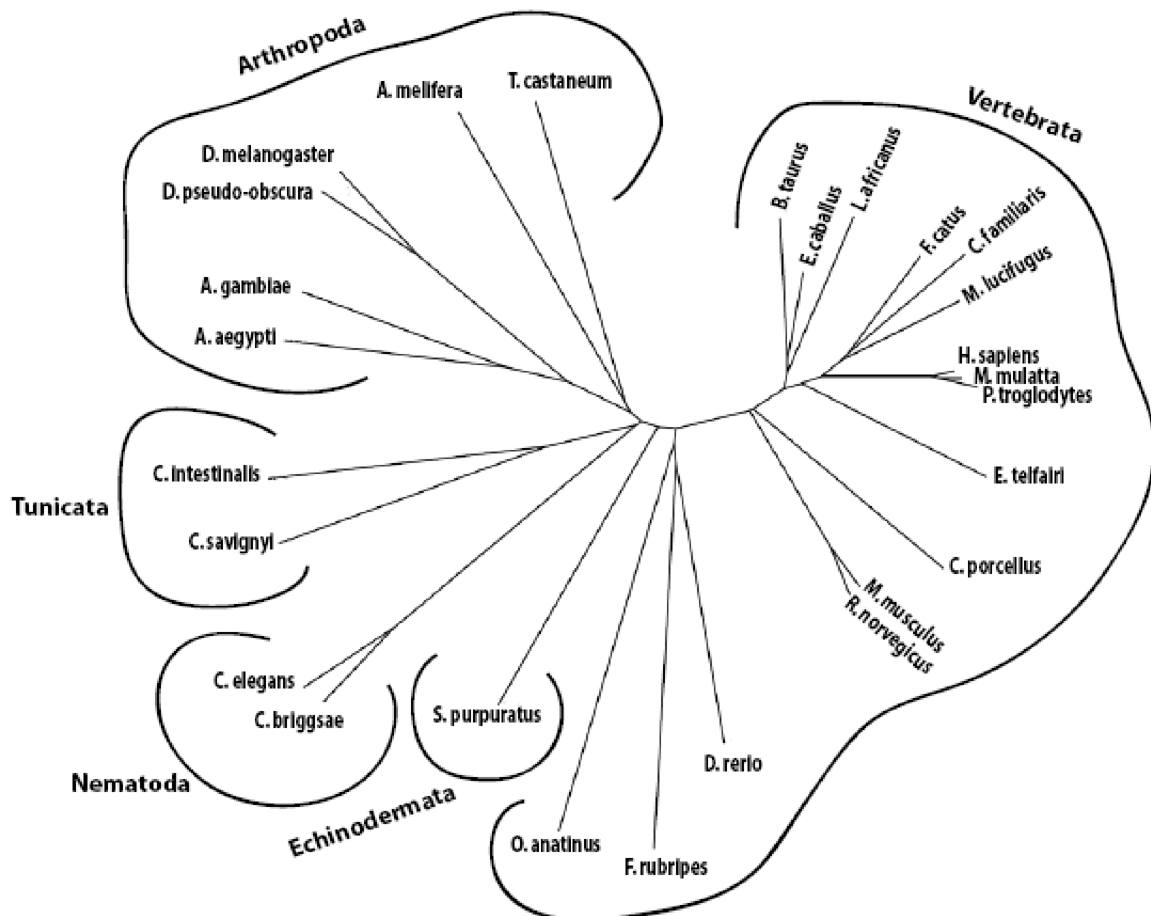


Fig. 1. *LEM3 is an evolutionary conserved LEM-domain containing protein. (A) Comparison of the predicted domain organization of LEM3 orthologues from Homo sapiens (hs), Mus musculus (mm), Rattus norvegicus (rn), Drosophila melanogaster (dm) and Caenorhabditis elegans (ce). Ankyrin repeats are shown in green, the LEM domain in red and a C-terminal domain of unknown function in violet. (B) Phylogenetic analysis of various LEM3 orthologues of major metazoan clades depicted as unrooted tree diagram.*

LEM3 mRNA is alternatively spliced and expressed in a tissue-restricted manner

Semiquantitative RT-PCR analysis of cDNAs derived from various human and mouse tissues, revealed highest expression levels of LEM3 mRNA in hematopoietic tissues including bone marrow, thymus and spleen (Fig. 2A). No or only low levels of LEM3 mRNA were detected in all other tested tissue samples (Fig. 2A). LEM3 is also highly expressed in cell lines derived from B-cell lymphomas (i.e. DAUDI, RAJI, RAMOS) and in a T-cell acute leukemia derived cell line (JURKAT), while no or only minor levels were detected in all investigated carcinoma-derived cell lines, in an erythroleukemia cell line (K-562) (data not shown), in a plasma cell leukemia derived cell line (ARH-77) and in peripheral blood monocytes obtained from two healthy donors (PBM) (Fig. 2A). These results confirm data in the NCBI EST and Unigene databases (http://www.ncbi.nlm.nih.gov/), reporting hANKRD41 expression primarily in lymphoid tissues and lymphoma. Interestingly, expression of LEM3 was not found to be restricted to cells of the lymphoid lineage during murine embryonal development as fibroblasts derived from mouse and rat embryos showed moderate levels of LEM3 transcript.

PCR analyses amplifying the 3' half of the LEM3 cDNA from human and mouse bone marrow cDNA libraries, produced a major DNA fragment of the expected size for a "full length" LEM3, as well as two minor smaller fragments in both human (Fig. 2B) and mouse samples (data not shown). Sequencing the smaller fragments revealed deletions of 75 or 264 base pairs, respectively, at position 1122 in the hLEM3 coding sequence. The longest transcript (termed LEM3α) was clearly the predominant isoform, while the two smaller isoforms, termed LEM3β and LEM3γ, were only weakly expressed. Interestingly, the smaller isoforms lack the second half of the LEM domain (LEM3β; Δ aa 375-400) or additionally parts of the common C-terminus (LEM3γ; Δaa 375-463).

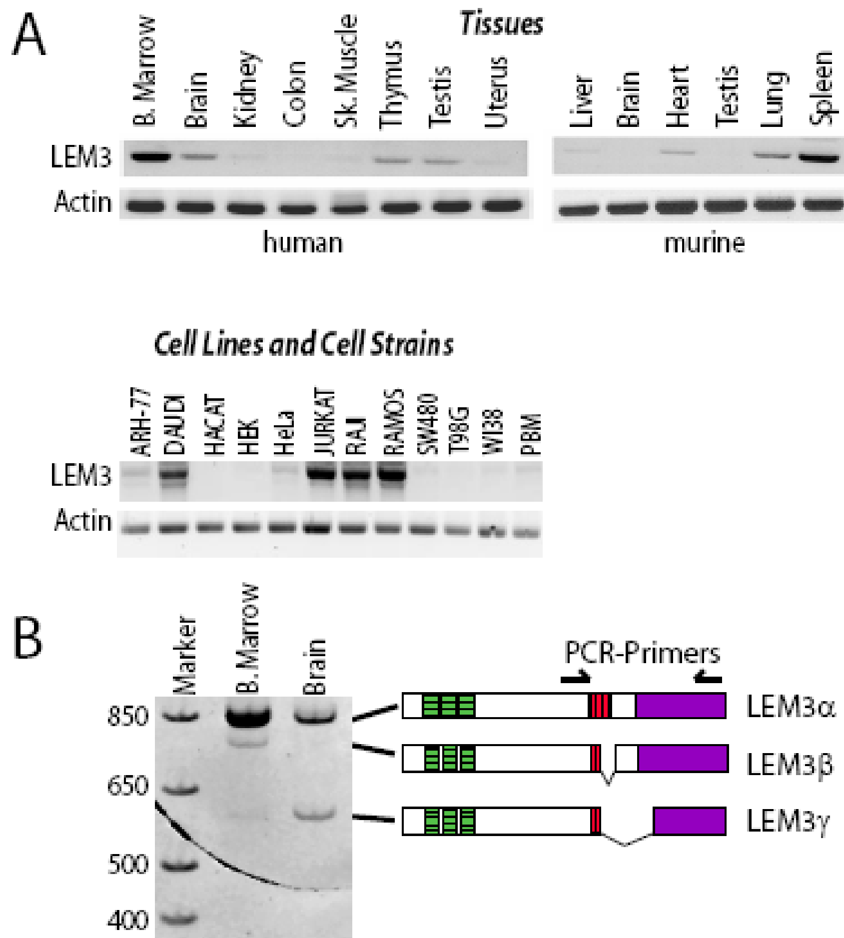


Fig. 2. *LEM3 is alternatively spliced and expressed in a tissue-restricted manner. (A) Semiquantitative RT-PCR analysis of LEM3 mRNA levels and of actin mRNA as control. B.Marrow (bone marrow), Sk.Muscle (skeletal muscle), PBM (peripheral blood monocytes). (B) DNA fragments of alternative LEM3 splice products were amplified by RT-PCR using primers shown in the schematic drawing, and analyzed by PAGE. B.Marrow (bone marrow).*

Human and murine LEM3 have different cellular localization

In order to determine the subcellular localization of LEM3α, we transiently expressed GFP- and V5-tagged versions of human and murine LEM3α in rat embryonic (oREC) cells. Interestingly, the localization of human and mouse LEM3α differed. Human LEM3α was localized predominantly in the cytoplasm, revealing a filamentous pattern in methanol fixed specimen, independent of the the nature, size and localization of the tag (Fig 3A). hLEM3β behaved like LEM3α (Fig.3, hLEM3β-V5), indicating that this localization does not require an intact LEM domain. The filamentous pattern of hLEM3α suggested colocalization with a major cytoskeletal network. Coimmunostaining analysis revealed that hLEM3α colocalized with the actin cytoskeleton, particularly with stress fibers, but not with microtubules or vimentin (Fig. 3B, C). Destroying actin filaments by Cytochalasin B treatment for 16 hours lead to aggregation of actin and reorganization of LEM3 (Fig. 3B). We concluded that hLEM3α associates with the cytoplasmic actin cytoskeleton. Similar results were observed for ectopically expressed murine mLEM3α (data not shown), although a major portion of mLEM3α accumulated in the nucleus (Fig. 3A, mLEM3-V5). The nuclear protein was distributed uniformly in the nucleoplasm and in speckle-type structures, where it colocalized with proteins involved in RNA-processing, including SC-35 and U-snRNPs (see supplementary Fig. 1A).

The different localization of human and mouse LEM3α was an intrinsic property of the proteins and independent of the cell systems, since expression of human and murine LEM3α in other mouse (C2C12) or human cells (U2OS, MCF7) revealed identical results (data not shown).

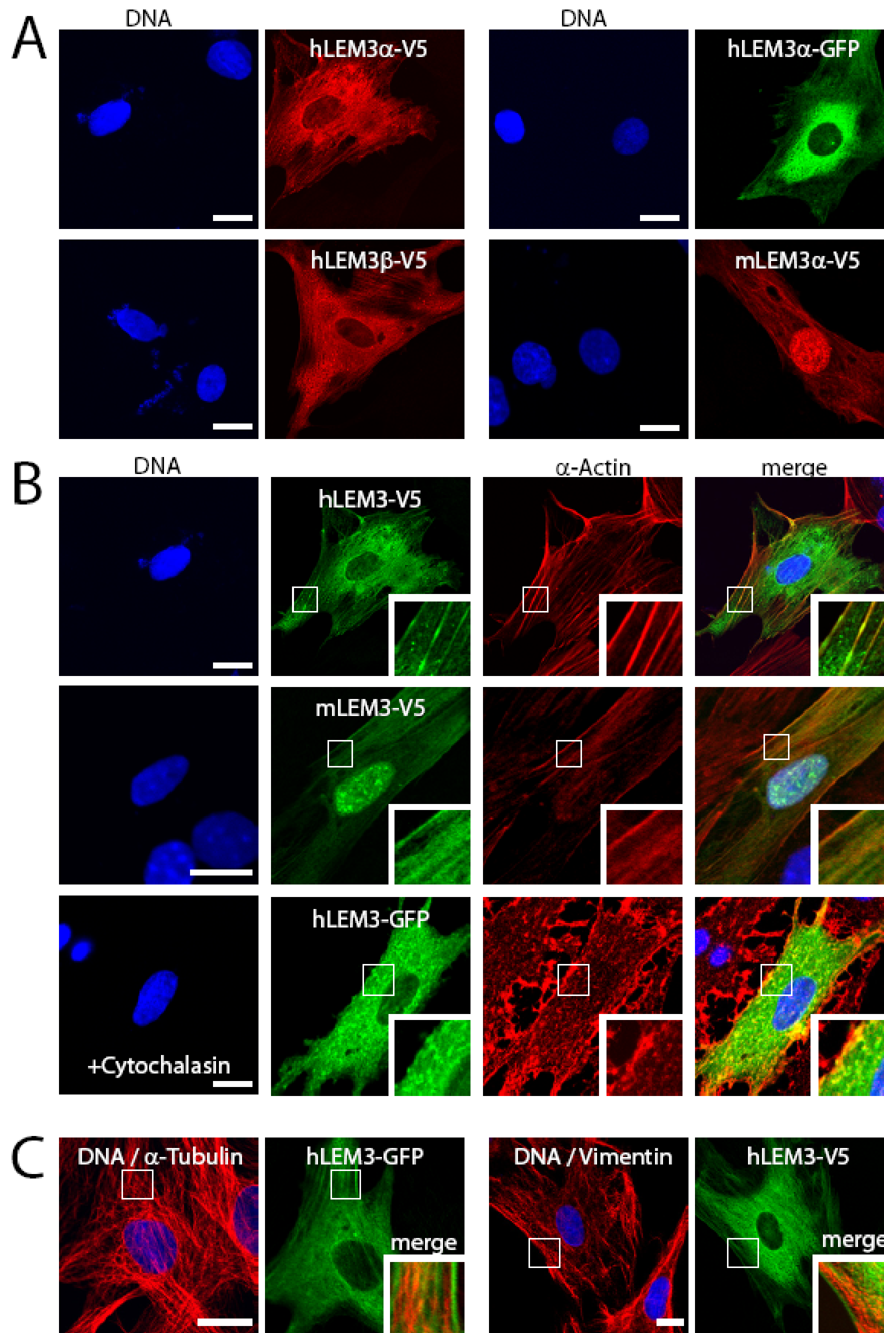


Fig. 3. *Ectopic human LEM3α and β localize in the cytoplasm, while mouse LEM3 is predominantly nuclear. (A) oREC cells transiently expressing human V5- or GFP-tagged hLEM3α or β or mLEM3α were fixed and processed for immunofluorescence microscopy using antibodies to V5 (red) and DAPI to visualize DNA (blue). LEM3α-GFP was viewed directly. (B) Untreated oREC cells expressing human or mouse LEM3α were stained with V5 (green) and α-Actin (red) antibodies as well as DAPI or oREC cells expressing LEM3α-GFP were treated with Cytochalasin (2µM for 16h), fixed and stained for α-Actin (red) and DNA (blue). (C) oREC cells expressing ectopic hLEM3-GFP or hLEM3-V5 were costained for α-Tubulin (red) or Vimentin (red) and DNA (blue). Confocal images are shown. Bars = 10µm.*

hLEM3 shuttles between the nucleus and the cytoplasm

Computational analyses revealed several putative nuclear export sequences (NES), as well as nuclear localization sequences (NLS) throughout hLEM3α (Fig. 4A), indicating that the protein shuttles between the nucleus and cytoplasm. To test this possibility, we treated hLEM3α expressing cells with Leptomycin B, a specific inhibitor of Crm-dependent nuclear export. Intriguingly, hLEM3α accumulated in the nucleus upon Leptomycin B treatment, often forming dot like structures in the nucleoplasm (Fig. 4B, supplementary Fig. 1B), indicating nucleo-cytoplasmic shuttling of the protein.

To investigate the contributions of different hLEM3α domains to nucleo-cytoplasmic shuttling, we tested the effect of Leptomycin B on the localization of expressed hLEM3α fragments. GFP fusions of C- or N-terminally truncated hLEM3α lacking the highly conserved GIY-YIG motif (ΔCT) or the ankyrin repeats (ΔANK), respectively, localized to the cytoplasm in the absence of Leptomycin B and accumulated in the nucleus in the presence of Leptomycin B, but did it less efficiently than full length LEM3α (Fig. 4B), suggesting that the C- and N-terminal domains of hLEM3α are both involved in the regulation of nucleo-cytoplasmic transport. The ectopically expressed LEM3α C-terminal fragment alone fused to GFP (CT), which has a molecular mass of 52 kD and is thus unable to passively diffuse through nuclear pore complexes, localized exclusively to the nucleus independent of Leptomycin B confirming the existence of functional NLS within the C-terminus. In contrast, a central region of hLEM3α lacking both the N-terminal ankyrin repeats and the C-terminal GIY-YIG domain (ΔANKΔCT), was excluded from the nucleus in the absence of Leptomycin B and was only weakly, possibly involving passive diffusion, translocated to the nucleus in the presence of Leptomycin B. Therefore we concluded that a non-canonical nuclear export signal might be located between the N-terminal ankyrin repeats and the LEM domain.

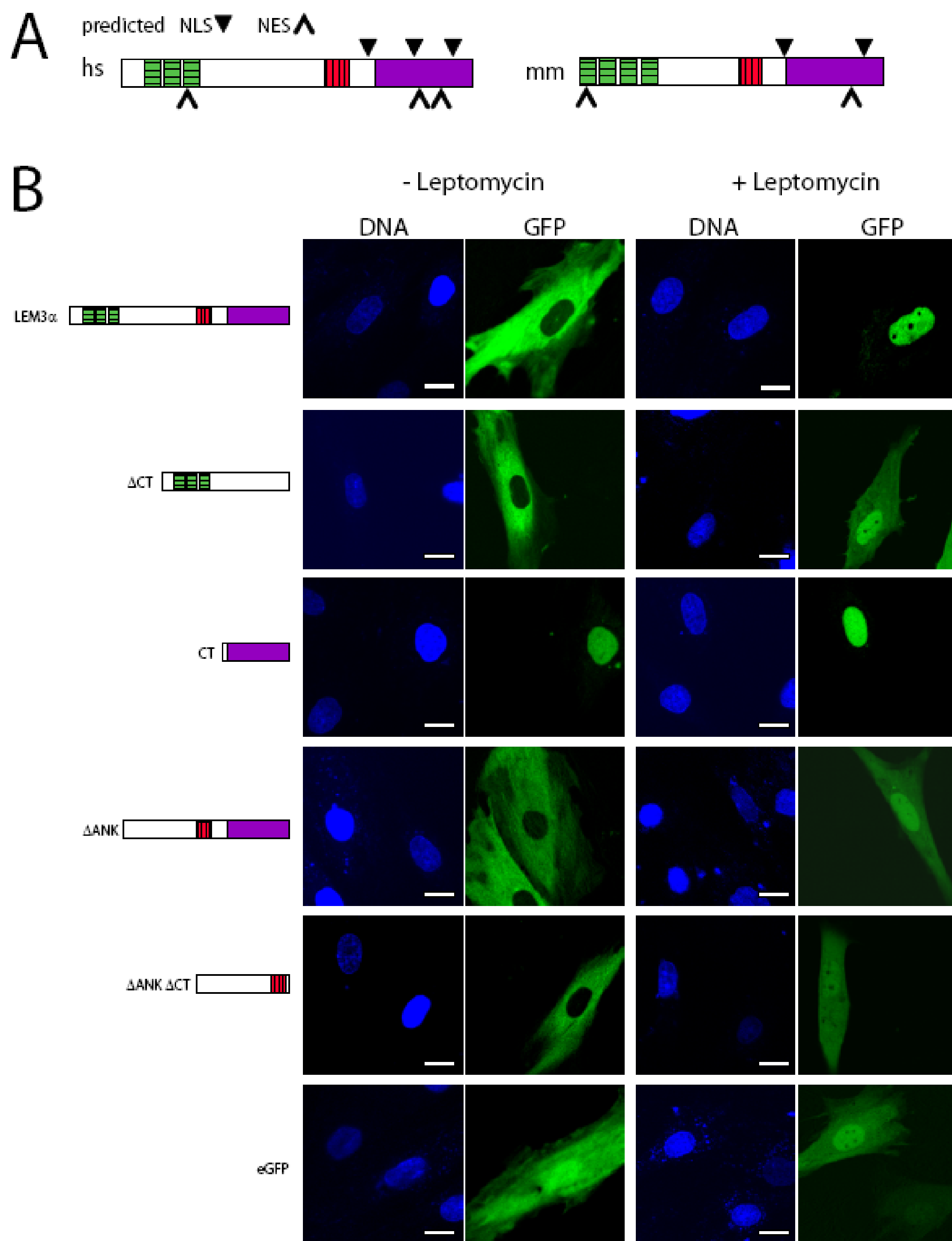


Fig. 4. *LEM3 is exported from the nucleus via a Crm-dependent pathway. (A) Localization of predicted NLS and NES signals in human (hs) and mouse (mm) LEM3α. Fluorescence analysis of living oREC cells expressing GFP-hLEM3α and stained with DAPI before and after treatment with Leptomycin B (5 ng/mL for 3 hours). (B) Live cell imaging of oREC cells expressing various human LEM3-GFP deletion constructs or eGFP as negative control before (left column) and after 3 hours of Leptomycin treatment (right column). Cells were stained with DAPI to visualize DNA (blue). In drawings, ankyrin repeats are shown in green, the LEM3 domain in red and the C-terminal motif in violet. Confocal images are shown. Bars, = 10µm.*

LEM3 binds BAF and this binding requires an intact LEM domain

Next, we investigated whether the LEM domain in LEM3α is able to interact with BAF, as previously demonstrated for all tested LEM domains (Dechat et al., 2004; Lee et al., 2001; Mansharamani and Wilson, 2005; Shumaker et al., 2001). Sequence alignments of the LEM domain of hLEM3α with those of other known human LEM proteins revealed that all residues previously identified by mutation analysis to be essential for BAF binding (Shumaker et al., 2001) were highly conserved in hLEM3α (Fig. 5A, brown boxes). We performed co-immunoprecipitation assays in order to test whether LEM3α and BAF are present in the same protein complex. Ectopically expressed V5-tagged human or mouse LEM3α or β isoforms were precipitated from cell lysates using anti-V5 antibodies, and the presence of BAF in the precipitate was tested by immunoblotting, using BAF antibodies. All ectopically expressed LEM3 proteins were efficiently precipitated by anti-V5 coupled protein G-beads (Fig. 5B, lower panel). Significant amounts of BAF co-precipitated with human and mouse LEM3α while hLEM3β, which misses part of the LEM domain, did not pull down BAF above background levels similar to control beads (Fig. 5B, upper panel, P-Co). hLEM2 used as a positive control pulled down BAF efficiently (Fig. 5B). Interestingly, precipitated BAF split up into three proteins with different molecular weights between 12 and 30kD. We speculate that formation of LEM-domain/BAF complexes may change BAF conformation (Forne et al., 2003) which are subsequently crosslinked by artificial oxidation during the precipitation procedure. These higher molecular weight complexes may not be destroyed during SDS-PAGE (also observed by (Dechat et al., 2004) (Zheng et al., 2000)). Recently it was also shown that monomeric BAF does not form complexes with the LEM motif (Cai et al., 2007).

We concluded that LEM3α associates with BAF and that an intact LEM domain is required for that association.

Despite the observed association of hLEM3α and BAF at biochemical level, immunofluorescence microscopy revealed mostly non-overlapping localization of hLEM3α and BAF in the cytoplasm and nucleus, respectively (Fig. 6, untreated). To test whether hLEM3α may affect BAF, when temporarily forced into the nucleus, we investigated BAF localization after treatment of hLEM3α-expressing oREC cells with Leptomycin B. Following Leptomycin B treatment for three hours, both hLEM3α and BAF were predominantly nuclear without any obvious effect on BAF distribution in ~50% of transfected cells (Fig. 6, normal phenotype). About 50% of transfected cells showed nuclear hLEM3α and a partial mislocalization of BAF to cytoplasmic aggregates, in addition to the nucleoplasmic distribution (minor delocalization phenotype). In contrast, non-transfected cells showed normal nucleoplasmic localization of BAF

after Leptomycin B treatment in ~95% of cases (Fig. 6, left graph), indicating that expression of hLEM3α combined with Leptomycin B treatment significantly affected BAF localization.

We next released Leptomycin B treated cells from the drug, allowed the cells to recover for five hours in normal growth medium and tested BAF localization. This treatment produced three different localization patterns of BAF. Only <5% of transfected cells had normal nucleoplasmic distribution of hLEM3α and BAF, ~45% had partial delocalization of BAF to cytoplasmic aggregates (medium phenotype), and ~55% of cells had severe delocalization of BAF (strong phenotype) (Fig. 6). In the latter cells, hLEM3α localized to the nucleus and the cytoplasm, indicating that translocation of hLEM3α back from the nucleus to the cytoplasm following release of Leptomycin B treatment caused redistribution of BAF. In the absence of ectopic hLEM3α expression BAF was unaffected and predominantly nuclear both in the presence of Leptomycin B or following its removal (Fig. 6, bar graphic) suggesting that BAF mislocalization is specifically caused by ectopically expressed hLEM3α. These data also confirm that hLEM3α and BAF can interact *in vivo*.

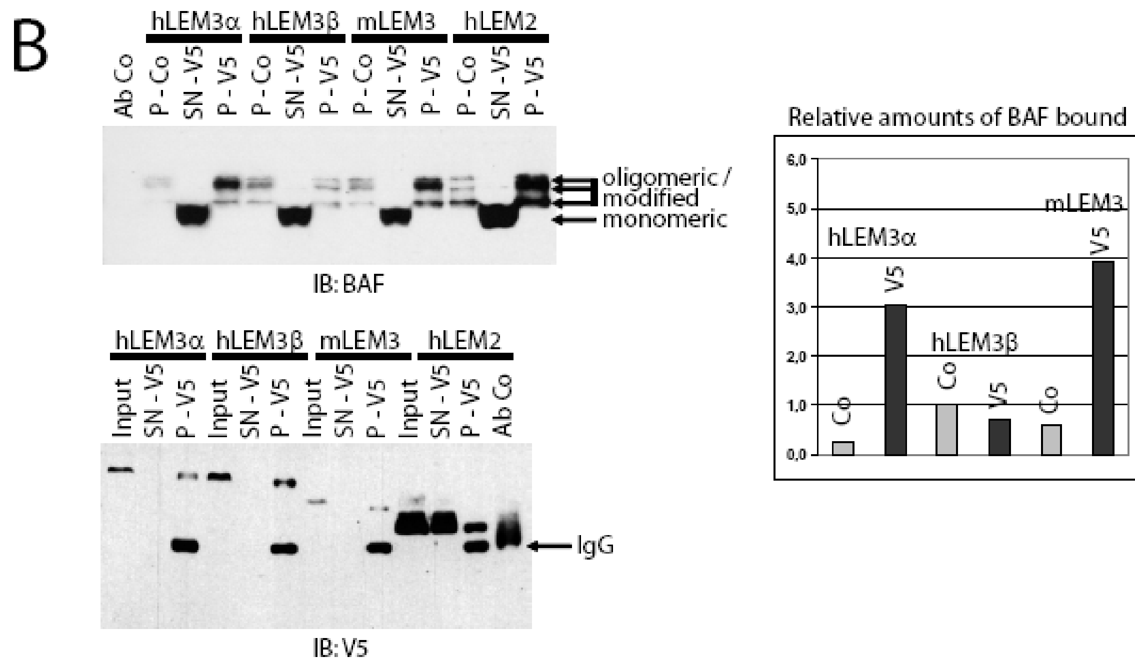


Fig. 5. *LEM3 interacts with BAF in a LEM domain-dependent manner. (A) Sequence alignment of characterized human LEM proteins, indicating conserved residues in blue (conservative substitutions) or red (identical residues). Residues essential for BAF binding according to Shumaker et al. (Shumaker et al., 2001)(Shumaker et al., 2001)(Shumaker, Lee et al. 2001)(Shumaker, Lee et al. 2001)(Shumaker, Lee et al. 2001)(Shumaker, Lee et al. 2001) are highlighted by coloured background. Amino acid positions in the protein sequence are indicated. (B) Expressed V5-tagged LEM2, hLEM3α and β and mLEM3 were immunoprecipitated from whole cell lysates using V5 antibodies and input, supernatant (SN-V5) and pellet (P-V5) fractions were analyzed by immunoblotting using antibodies to BAF (upper panel) and V5 (lower panel). P-Co shows control precipitates in the absence of antibodies. P fractions are 6 times more concentrated than SN and I fractions. Antibody control (Ab Co) shows 100ng of V5 antibody. The relative amounts of BAF in V5- and control precipitates were measured by determining the intensity of bands using ImageQ., P (pellet), SN (supernatant). Note that only LEM isoforms with an intact LEM motif precipitate BAF.*

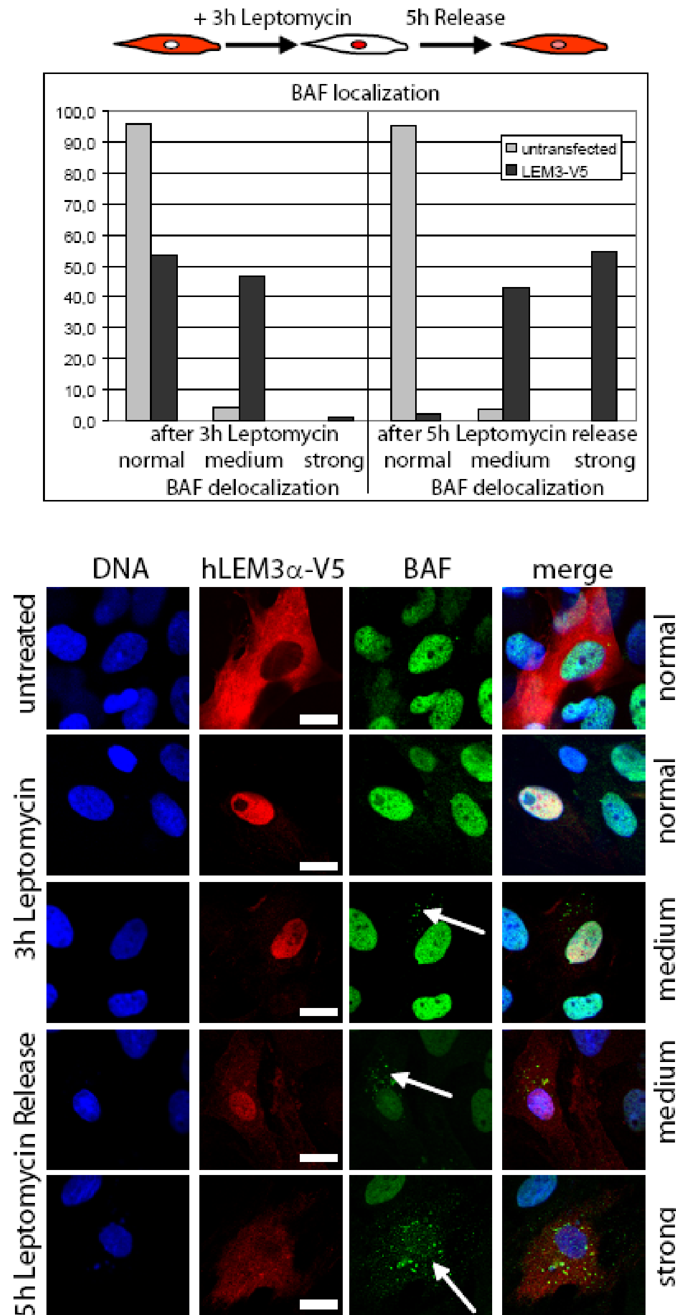


__Fig. 6.__ Expressed hLEM3 affects BAF localization upon transient nuclear accumulation. oREC cells expressing hLEM3α-V5 were treated with Leptomycin for 3 hours and released from the drug for 5 hours. Cells were fixed at each time point and processed for immunofluorescence microscopy using antibodies to V5 (red), BAF (green) and DNA (blue). Confocal images are shown. Arrows indicate cytoplasmically mislocalized BAF. Bar, 10µm. Cells (n>200 each timepoint) were scored for medium or strong mislocalization of BAF and untransfected cells on the same plate were used as a negative control (normal). Bar diagram shows percentage of cells with "normal", "medium" or "strong" BAF delocalization phenotype after 3 hours of Leptomycin treatment (left side) and after 5 hours of Leptomycin release (right side).

LEM-3 is not required for viability in embryos or adults

Next, we aimed at testing the physiological relevance of hLEM3 function *in vivo* by RNA interference-mediated downregulation of LEM3 in mammalian cells. However RNAi turned out to be very inefficient in cells that express significant levels of endogenous LEM3 mRNA (JURKAT and RAMOS) due to unspecific, transfection-induced cell death and extremely low transfection efficiencies. We therefore switched to a different organism, *C. elegans*, where *lem-3* is expressed and RNAi mediated downregulation can be efficiently done *(lem-3(RNAi))* (Lee, Gruenbaum et al., 2000). RT-qPCR analysis revealed that *lem-3(RNAi) caused* a significant reduction in *lem-3* transcript levels (Fig. 7A, inset). We next examined animals with reduced levels of LEM-3 for any phenotype during development. We found that there was no increased embryonic lethality; *lem-3(RNAi)* embryos developed at normal rates into fertile adult nematodes with a normal brood size (Fig. 7A). These *lem-3(RNAi)* adults had no detectable phenotype, displayed normal feeding behavior, and produced viable fertile offspring as compared to control animals fed with L4440 feeding vector (data not shown). Nuclear localization of other nuclear envelope proteins was not dependent on LEM-3. Neither the localization of Ce-lamin and Ce-emerin nor that of GFP-BAF-1 (Margalit et al., 2007b) were affected by the knockdown of *lem-3* (Fig. 7B). We concluded that LEM-3 is not essential and is not required for the nuclear envelope localization of Ce-lamin, Ce-emerin or BAF-1.

Given the functional redundancy, reported for the other two worm LEM-domain proteins, LEM-2 and Ce-emerin (Liu et al., 2003), we tested the effect of *lem-3(RNAi)* on embryonic lethality and brood size of worms homozygous for deletion in *emr-1* (VC237) or *lem-2* (tm1582). Applying *lem-3(RNAi)* to the deletion strains had no affect on embryonic lethality (Fig. 7A) or the brood size of the adult animals, similar to RNAi with the L4440 control vector (data not shown). The strain homozygous for *lem-2* deletion and heterozygous for the *emr-1* deletion shows 70-80% embryonic lethality. Again, down regulating lem-3 did not increase embryonic lethality (Fig. 7A). We concluded that there is no obvious functional redundancy between LEM-3 and the other *C. elegans* LEM domain proteins.

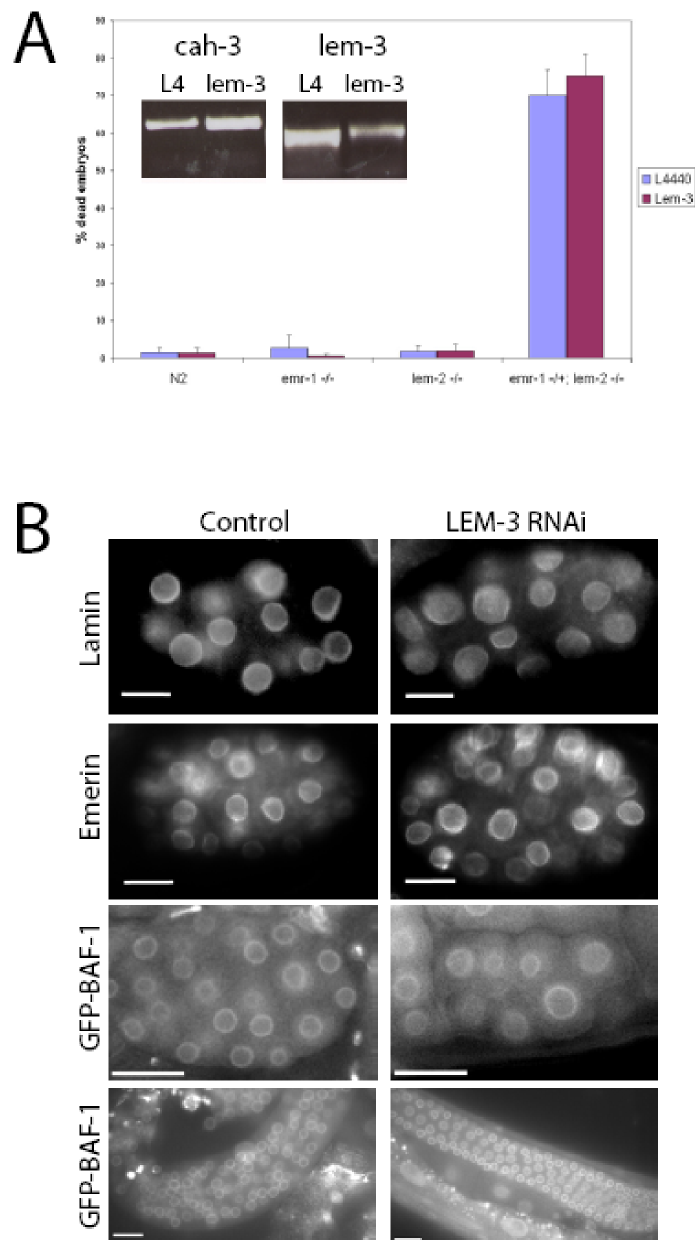
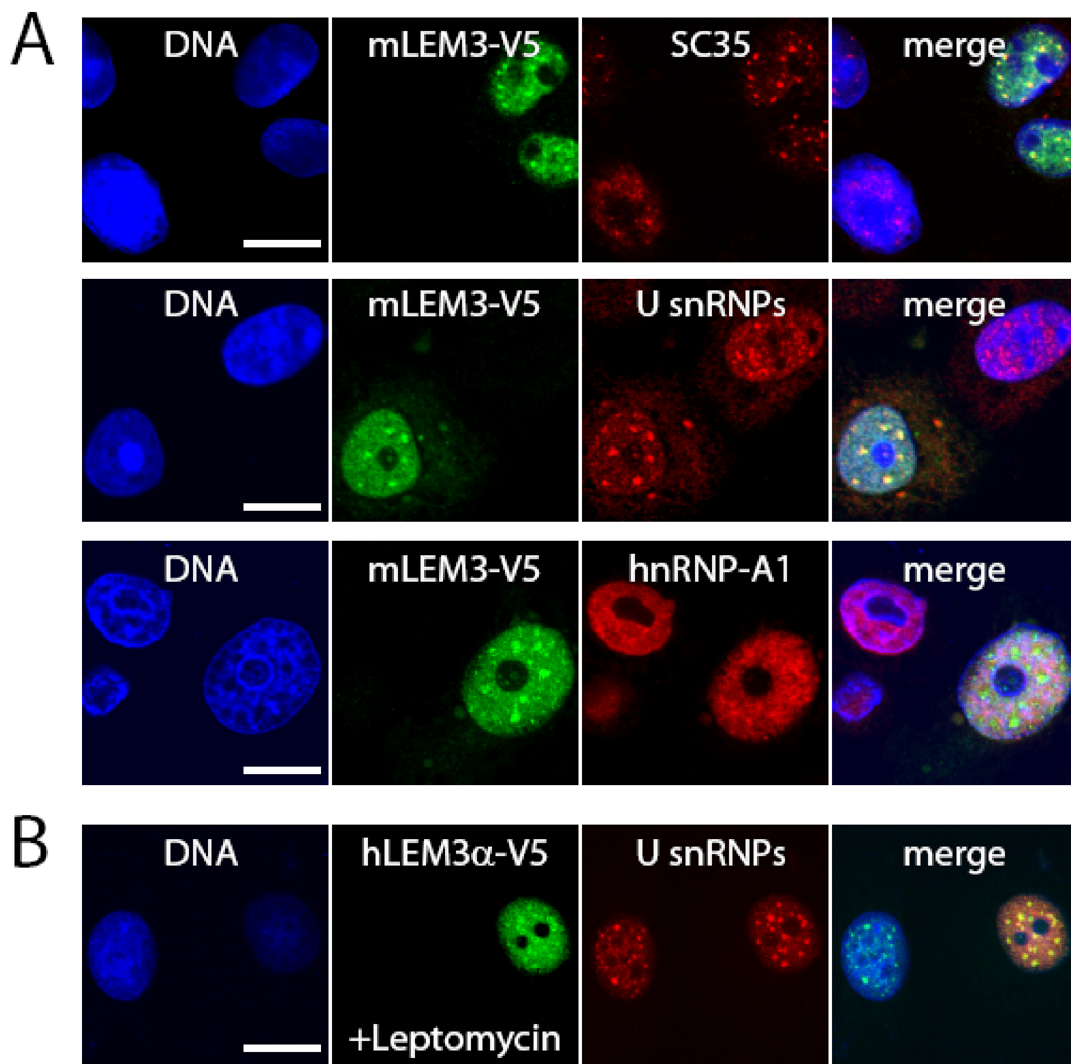


Fig. 7. *Downregulation of lem-3 does not cause embryonic lethality in wildtype (N2), emr-1-/emr-1⁻ (emerin null), lem-2⁻/ lem-2⁻ (LEM-2 null) or lem-2⁻/ lem-2-; emr-1⁻/+ (LEM-2 null and heterozygous for emerin) worms. Strains were fed with bacteria expressing either a control vector L4440 (blue) or lem-3 dsRNA (purple). The graph shows the percentage of embryonic lethality as Mean and SD. Insert shows RT-qPCR analysis of RNA from worms fed with control vector L4440 (L4) or lem-3. The carbonic anhydrase cah-3 gene was used as a control for RNA quality and amounts. RT-qPCR showed a significant reduction in lem-3 RNA levels following lem-3(RNAi). (B) Downregulation of LEM-3 did not affect the localization of Ce-lamin, Ce-emerin, or GFP-BAF-1. Worms were fed with bacteria containing the L4440 vector (control) or lem-3(RNAi) and the offspring embryos were stained by indirect immunofluorescence using antibodies against Ce-lamin (Lamin) or Ce-emerin (Emerin). GFP-BAF-1 was viewed directly in live embryos. Bars = 5 µm.*



Supplemental Fig. 1. *LEM3 colocalizes with splicing speckles in the nucleus. (A) mLEM3-V5 was expressed in oREC and cells were processed for immunofluorescence microscopy using antibodies to V5 (green), and either SC35 (red), U snRNPs (red) or hnRNP-A1 (red) and DNA (blue). (B) oREC cells expressing hLEM3-V5 were treated with Leptomycin for 3 hours, fixed and stained for V5 (green), U snRNPs (red) and DNA (blue). Confocal images are shown. Bars = 10µm.*

Discussion

The availability of whole genome sequences from many organism and improved computational tools have lead to the identification of novel putative LEM-domain proteins in various species, such as LEM3, 4 and 5 in mammals (Lee and Wilson, 2004; Yuki et al., 2004), and Bocksbeutel (Wagner et al., 2004) and Otefin (Padan et al., 1990) in *Drosophila*. Besides their common feature, the presence of a LEM motif and the interaction with BAF, many of the LEM proteins also have unique functions in chromatin organization, gene expression, and signaling (reviewed in (Schirmer and Foisner, 2007). LAP2β interacts with HDAC3 (Somech et al., 2007), emerin, MAN1, and LAP2α interact with transcription factors or repressors, such as GCL, Lmo7, Btf and Crx and Rb (Dorner et al., 2006; Haraguchi et al., 2004; Holaska et al., 2003; Holaska et al., 2006; Mansharamani and Wilson, 2005; Melcon et al., 2006; Wilkinson et al., 2003), emerin and MAN1 bind to and affect β-catenin and Smad-signaling components, respectively (Markiewicz et al., 2006; Osada et al., 2003; Pan et al., 2005). Not surprisingly an increasing number of human diseases has been linked to genetic mutations in genes encoding LEM proteins in the past years (Vlcek and Foisner, 2007; Wagner and Krohne, 2007).

In this report we describe the initial biochemical and cell biological characterization of one of the novel LEM-domain proteins, termed LEM3 or ANKRD41 (Lee and Wilson, 2004). We show that the LEM domain in LEM3 is functional in binding to BAF, which confirms that the protein is a bona fide LEM protein. In addition, we also demonstrate various intriguing new and unique features of LEM3 within the LEM proteins: (A) LEM3 mRNA expression is mostly restricted to hematopoietic tissues, while most of the characterized mammalian LEM proteins are widely expressed (Brachner et al., 2005; Ellis et al., 1998; Lin et al., 2000; Theodor et al., 1997). (B) LEM3 molecular domain organization is clearly distinct from that of other members; its LEM domain is localized in the middle of the polypeptide, and it contains ankyrin repeats at the N-terminal domain and a GIY-YIG motif at its C-terminal end. (C) LEM3 encodes smaller isoforms that lack a functional LEM domain and cannot bind BAF. Thus, LEM3 is so far the only LEM gene encoding isoforms that can or cannot bind BAF, indicating a potential role of LEM3 in BAF regulation or vice versa. (D) The most intriguing feature of LEM3 within the LEM family is probably its unique cellular localization. Whereas all characterized members of the LEM protein family are localized in the nucleus and except for LAP2α and ζ isoforms are anchored in the inner nuclear membrane (Wagner and Krohne, 2007), LEM3 is localized both in the nucleoplasm and cytoplasm and this localization depends on cell condition and species. Our data strongly suggest that LEM3 shuttles between the nucleus and cytoplasm and that shuttling is intrinsic to the protein and independent of the cell type; hLEM3 is effectively exported from the

nucleus and localizes to actin fibers, while mLEM3 tends to accumulate in the nucleus. We speculate that a mouse sequence-specific Leucin to Tryptophan substitution in a putative NES in the human protein at position 115 might be responsible for the different behaviour (Kutay and Guttinger, 2005).

What may be the function of LEM3?

We found that hLEM3α, when forced to accumulate in the nucleus by transiently inhibiting nuclear export, caused BAF displacement from diffused nucleoplasmic distribution into cytoplasmic aggregates. As hLEM3α did not colocalize with cytoplasmic BAF aggregates we speculate that interaction between both proteins might be restricted to the nucleus. Interaction between LEM3α and BAF might be only transient or could be regulated by post-translational modifications of hLEM3 or BAF in the nucleus (data not shown).

In silico analyses revealed a high degree of conservation in domain organization of LEM3 orthologues from *H. sapiens, M. musculus*, *D. melanogaster*, to *C. elegans*. The highest level of conservation was detected in the C-terminal region of the molecule, containing the GIY-YIG motif, indicating important functions of this domain. The GIY-YIG motif is related to the conserved COG3680a domain present in bacterial or phage proteins with endonuclease and/or recombinase activities (Dunin-Horkawicz et al., 2006; Van Roey et al., 2002). It is tempting to speculate, therefore, that LEM3 may also be involved in cellular processes involving nuclease activities. While bacterial GIY-YIG endonucleases also contain an additional DNA-binding, mostly a zinc-finger domain, LEM3, which misses a predictable zinc finger domain, could bind to DNA via the interaction of its LEM domain with BAF. Following this model, LEM3 splice isoforms lacking the LEM domain function are predicted to exert inefficient DNA binding and nuclease activity. Consistent with a function of LEM3 in nuclease-involving pathways is the observed association of LEM3 with RNA-processing factories (supplementary figure), which are known to be functionally linked to double-strand break DNA-repair complexes (Campalans et al., 2007; Herrmann et al., 2007). Intriguingly, BAF-emerin and BAF-LAP2α complexes (Jacque and Stevenson, 2006; Suzuki et al., 2004; Van Maele et al., 2006) were also found to be involved in the integration of viral DNA into the host DNA, which also involves double strand breaks. In view of the high expression levels of LEM3 in B-cells, it is tempting to speculate that LEM3 and BAF are predominantly involved in DNA repair/recombination pathways during B-cell development. Interestingly, transcript levels of LEM3 are decreased upon long-term stimulation of RAMOS B-cells with anti-human IgM antibodies (A. Brachner, unpublished data), consistent with a function of LEM3 during B-cell differentiation. Smaller LEM3 isoforms, hLEM3β and

hLEM3γ, which miss a functional BAF-binding LEM motif may further regulate this activity (data not shown).

Another potential function of LEM3 may be mediated by the ankyrin repeats, located in the N-terminus of LEM3. The ankyrin repeat (AR) is one of the most abundant protein motifs, found in more than 400 different human proteins (Mosavi et al., 2004; Sedgwick and Smerdon, 1999). The number of repeats within a stretch of ankyrin motifs can range from one to over 30, but two to six consecutive repeats seem to be most common (Mosavi et al., 2004). Generally, ARs are thought to be universal platforms for protein-protein interaction, and proteins containing ankyrin repeats are involved in a multitude of cellular processes including signaling, transcription, inflammation and development (Mosavi et al., 2004). Interaction partners of the AR in LEM3 are not known yet.

LEM-3 was also identified in a recent RNAi-screen for genes involved in axon guidance in *C.elegans* (Schmitz et al., 2007). Based on the detection of LEM3 EST sequences in murine sympathetic ganglion (NCBI Unigene database) and a moderate expression observed in human brain shown here, a role of LEM3 in neuronal development can also be envisaged.

Materials and Methods

Cell culture and reagents

HeLa cells and the primary embryonic rat cell clone oREC (Cerni et al., 1990) were routinely cultivated in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, USA), 100 U/mL Penicillin/Streptomycin and 2 mM L-Glutamine at 37°C in a humidified atmosphere containing 8,5% CO_2. Transient transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). Pharmacological intervention of Crm-dependent nuclear export was done by treatment of cells with 10 ng/mL Leptomycin B (Sigma, Munich, Germany) in complete growth medium for 3 hours**.**

PCR analysis

Total RNA was purified from cell lines by standard techniques or purchased as total RNA collections of human and mouse tissues (Human and mouse total RNA Master Panel II, Clontech, Palo Alto, USA). Poly$(A)^+$-mRNA was extracted from total RNA using the mRNA-Isolation Kit and reverse transcribed using the first-strand cDNA Synthesis Kit (both from

Roche, Mannheim, Germany). Aliquots of the resulting products were used as templates for PCR-amplifications using the Go-Taq PCR Kit (Promega, Mannheim, Germany) and primers as listed in Supplementary Table 1. cDNA levels were normalized for actin expression levels.

Primers used for determination of expression	**Sequence 5' – 3'**
human, mouse, rat Actin forward	ATC TGG CAC CAC ACC TTC TAC
human, mouse, rat Actin reverse	CAG CCA GGT CCA GAC GCA GG
human LEM3 all isoforms forward	CCA GCC CGA GCC TTC TCA CTG AC
human LEM3 all isoforms reverse	CGC TCG CCT TCA GCC AGG AAG AC
mouse LEM3 all isoforms forward	ACT GGC GGA GGC ACT AAG GAC AGG
mouse LEM3 all isoforms reverse	CCG AGA GGG TGG CCA ATG GGC AAC
human LEM3 splice isoforms forward	TGC CTG TGG GAG CAC CAG ACA TC
mouse LEM3 splice isoforms forward	AAC CCG TAC TGC CTG GTG ATG G
rat LEM3 splice isoforms forward	ACC CTT ACT GCT TGG TGA TG
rat LEM3 splice isoforms reverse	TCA GCC TCG AGC CTG AAT GTC

Primers used for cloning	**Sequence 5' – 3'**
human LEM3 forward	CAC CGC TAG CAT GTG CTC GGA GGC CCG CCT GG
human LEM3 reverse	GTA TCT AGA GCC CCG GGC CTG GAT GTC
mouse LEM3 forward	CAC CTG GGA CAT GGC CGA TAC TGC ATG CTT GG
mouse LEM3 reverse	GGA GCC TCG AGC CTG AAT GTC CTG A

Table 1: Primers used for cloning and PCR analyses.

Antibodies

Mouse anti-V5 antibodies were purchased from Invitrogen (Carlsbad, USA); mouse anti-alpha-tubulin, anti-vimentin, and anti SC-35, and rabbit sera against actin and V5 from Sigma (Munich, Germany). Anti-LAP2α antibody was described previously (Vlcek et al., 2002), antiserum to BAF was a generous gift of K. Furukawa (Niigata University, Niigata, Japan; (Furukawa, 1999), the H20 antiserum generated against the cap structure of U snRNAs was kindly provided by R. Lührmann (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany (Bochnig et al., 1987); and the monoclonal antibody 4B10 recognizing hnRNP-A1 was generously provided by G. Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia, USA; (Choi and Dreyfuss, 1984)).

Plasmids and cloning strategy

Human and mouse full length LEM3 and splice variants were amplified by PCR using bone marrow cDNA and Pfx-polymerase (Invitrogen, Carlsbad, USA). For primers, see Table 1. PCR products were cloned either directly into pENTR/D-TOPO by Topoisomerase based cloning (Invitrogen) or cut with SalI and XbaI and cloned into NheI and XbaI sites of the peGFP-C1

vector (Clontech, Palo Alto, USA). For eukaryotic expression of V5 tagged proteins, hLEM3 was shuttled with the LR-recombination reaction (Invitrogen) into pTRACER-B, made Gateway®-compatible by insertion of the "conversion cassette" into the EcoRV site. Vectors expressing deletion mutants of hLEM3 were constructed by cloning PCR-generated hLEM3 fragments into peGFP-C1 using the same cloning strategy. The expression plasmid pTRACER-LEM2FL encoding full length human LEM2-V5 was described previously (Brachner et al., 2005).

Subcellular fractionation, gel electrophoresis and immunoblotting

To prepare total lysates, cells were scraped off, washed in cold PBS and resuspended in cold high-salt RIPA buffer (25 mM Tris-HCl pH 7.4, 500 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, "Complete" protease inhibitor mix (Roche, Mannheim, Germany)). For subcellular fractionation cells were incubated in hypotonic buffer H (10 mM HEPES pH 8.0, 10 mM KCl, 100 µM EDTA, 100 µM EGTA, 1 mM DTT) for 15 minutes on ice, NP-40 was then added to 0,5% final concentration and cells were vortexed for 10 seconds and nuclei spun down in a table centrifuge at 13.000 g for 1 minute. The supernatants containing cytoplasmic proteins, and nuclei in the pellet nuclei were washed in buffer H , resuspended in high salt RIPA and analyzed by SDS-PAGE and immunoblotting .

Co-Immunoprecipitation

Cells were scraped off the plates, washed in ice cold PBS plus 1mM $NaVO_4$ (Sigma, Munich, Germany) and lysed in CoIP lysis buffer (50 mM Tris-HCl pH 7.4, 4 mM $MgCl_2$, 10 mM β-Glycerophosphat, 150 mM NaCl, 0.1% SDS, "complete" protease inhibitor cocktail (Roche), 1% Triton X-100, 1 mM $NaVO_4$) on ice for 15 minutes. Insoluble material was removed by centrifugation at 300 g for 5 minutes. Supernatants were pre-cleared with BSA-adsorbed G-protein-coupled sepharose beads (Sigma, Munich, Germany) and incubated either with beads alone or beads coupled to monoclonal anti-V5 antibodies (Invitrogen, Carlsbad, USA) for 2 hours at 4°C. Immunoprecipitates were washed in lysis buffer, eluted in Laemmli sample buffer and analyzed by immunoblotting (Gerner et al., 2002).

Immunofluorescence and live-cell microscopy

Cells were grown on poly-L-lysine-coated glass coverslips, fixed in methanol at –20°C for 1 minute, or alternatively fixed in 4% paraformaldehyde for 10 minutes and permeabilized in PBS / 0.5% Triton X-100 for 5 minutes. Cells were blocked in PBS / 0.5% gelatine for 15

minutes, incubated with primary antibodies for 45 minutes, washed and re-probed with the appropriate secondary antibodies conjugated to either TexasRed, Cy-3, Cy-5 (Jackson Immuno Research, West-Grove, USA) or Alexa-488 (Molecular Probes, Leiden, The Netherlands) for 45 minutes. DNA was counter-stained with 100 ng/mL DAPI (Sigma, Munich, Germany) for 5 minutes, and samples were mounted in Mowiol (Fluka, Buchs, Switzerland) and viewed with a confocal laser scanning microscope (LSM-Meta, Zeiss, Jena, Germany) using a Plan-Apochromat 63x oil immersion objective (NA=1.40). Live-cell imaging was performed by seeding transfected cells into ibidi-treat® microscopy slides (Ibidi, Munich, Germany). Digital images were analyzed, adjusted for brightness and contrast, and mounted using the LSM-Image-Browser (Zeiss, Jena, Germany) and Adobe Photoshop (Adobe Systems Inc, San Jose, USA).

C. elegans strains, antibodies and RNAi mediated experiments

C. elegans N2 and VC237 strains were obtained from the *C. elegans* Genome Center and cultured as described previously (Brenner, 1974). tm1582 was obtained from Shohei Mitani, (Tokyo Women's Medical University School of Medicine, Tokyo). YG1002 strain expressing GFP-BAF-1 is described in (Margalit et al; 2007). Both the *emr-1* deletion strain, VC237, and the *lem-2* deletion strain, tm1582, were outcrossed three times. The strain homozygous for *lem-2* deletion and heterozygous for *emr-1* deletion was obtained using the VC237 and tm1582 strains. Clone I-4B14 (for lem-3 *RNAi*, MRC Gene Service) or the empty L4440 construct were used for RNAi feeding at 16°C as described (Fridkin et al., 2004). Worms were either examined live for viability and brood size or collected for RT-qPCR as described (Margalit et al., 2007). Worms were also fixed and stained for indirect immunofluorescence using the following antibodies: Rabbit anti-Ce-lamin sera 3932 or Rabbit anti-Ce-emerin sera 2570 were used at 1:100 dilution. Immunostained embryos and live worms expressing GFP constructs were imaged by using a Zeiss Axioplan II microscope equipped for fluorescence. The following primers were used for RT-qPCR: For *lem-3*, forward 5'-cctgcaattgctgctgtaaa-3', reverse 5'-gttggcttggatggtgtttt-3' or forward 5'-acatgaaatatcccacggag-3', reverse 5'-ccttccagcttcaaagttga-3'. For cah-3, forward 5'-cacttccattggggagagaa-3', and reverse 5'- acaacgcctttccctctttt -3'.

Computer-assisted analysis

Alignments of cDNA sequences and database searches were performed by NCBI-BLAST (http://www.ncbi.nlm.nih.gov/blast/), GraphAlign (http://darwin.nmsu.edu/cgi-bin/graph_align.cgi) (Spalding and Lammers, 2004) and ClustalW (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994). Genomic analysis was done using the

ENSEMBL-Genome Browser (http://www.ensembl.org/) (Sanger Institute). The sequences of hLEM3 orthologues were predicted using the GENSCAN software (http://genes.mit.edu/genscan.html) (Burge and Karlin, 1997). Protein motifs and pattern searches were performed using SMART (http://smart.embl-heidelberg.de/) (Letunic et al., 2004; Schultz et al., 1998), CDD (NCBI; http://www.ncbi.nlm.nih.gov/) and PSORT-II (http://psort.nibb.ac.jp/form2.html). Transmembrane domains were calculated using the membrane protein topology database (http://blanco.biomol.uci.edu/mptopo/) (Jayasinghe et al., 2001), the TMHMM 2.0 prediction software (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001), the SOSUI system (http://sosui.proteome.bio.tuat.ac.jp/) and the DAS-TMfilter algorithm (Cserzo et al., 2004). Phylogenetic tree predictions were visualized using the PhyloDraw software (Choi et al., 2000).

Acknowledgements

The authors would like to thank Chantal Rodgarkia-Dara for generous supply of materials as well as path breaking discussions! The authors are indebted to Kazuhiro Furukawa (Niigata University, Niigata, Japan), Reinhard Lührmann (Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany), Gideon Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia, USA) for their generous gifts of antibodies. and Shohei Mitani (Tokyo) for strains. We also thank the *C. elegans* Genetic Center (which is funded by the National Institutes of Health [NIH] National Center for Research Resources) for strains. This study was supported by grants from the Austrian Science Research Fund (FWF P17871) to RF and the EURO-Laminopathies research project of the European Commission (Contract LSHM-CT-2005-018690) to RF and YG.

Abbreviations

AR, Ankyrin repeat, BAF, Barrier-to-Autointegration Factor; LAP, Lamina-associated polypeptide; LEM, Lamina-associated polypeptide2-Emerin-MAN1; PAGE, polyacrylamide gel electrophoresis

References

Bochnig, P., Reuter, R., Bringmann, P. and Luhrmann, R. (1987). A monoclonal antibody against 2,2,7-trimethylguanosine that reacts with intact, class U, small nuclear ribonucleoproteins as well as with 7-methylguanosine-capped RNAs. *Eur J Biochem* **168**, 461-7.

Brachner, A., Reipert, S., Foisner, R. and Gotzmann, J. (2005). LEM2 is a novel MAN1-related inner nuclear membrane protein associated with A-type lamins. *J Cell Sci* **118**, 5797-810.

Burge, C. and Karlin, S. (1997). Prediction of complete gene structures in human genomic DNA. *J Mol Biol* **268**, 78-94.

Cai, M., Huang, Y., Ghirlando, R., Wilson, K. L., Craigie, R. and Clore, G. M. (2001). Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structures: one binds BAF and the other binds DNA. *Embo J* **20**, 4399-407.

Cai, M., Huang, Y., Suh, J. Y., Louis, J. M., Ghirlando, R., Craigie, R. and Clore, G. M. (2007). Solution NMR structure of the barrier-to-autointegration factor-Emerin complex. *J Biol Chem* **282**, 14525-35.

Campalans, A., Amouroux, R., Bravard, A., Epe, B. and Radicella, J. P. (2007). UVA irradiation induces relocalisation of the DNA repair protein hOGG1 to nuclear speckles. *J Cell Sci* **120**, 23-32.

Cerni, C., Patocka, K. and Meneguzzi, G. (1990). Immortalization of primary rat embryo cells by human papillomavirus type 11 DNA is enhanced upon cotransfer of ras. *Virology* **177**, 427-36.

Chen, I. H., Huber, M., Guan, T., Bubeck, A. and Gerace, L. (2006). Nuclear envelope transmembrane proteins (NETs) that are up-regulated during myogenesis. *BMC Cell Biol* **7**, 38.

Choi, J. H., Jung, H. Y., Kim, H. S. and Cho, H. G. (2000). PhyloDraw: a phylogenetic tree drawing system. *Bioinformatics* **16**, 1056-8.

Choi, Y. D. and Dreyfuss, G. (1984). Isolation of the heterogeneous nuclear RNA-ribonucleoprotein complex (hnRNP): a unique supramolecular assembly. *Proc Natl Acad Sci U S A* **81**, 7471-5.

Cserzo, M., Eisenhaber, F., Eisenhaber, B. and Simon, I. (2004). TM or not TM: transmembrane protein prediction with low false positive rate using DAS-TMfilter. *Bioinformatics* **20**, 136-7.

Dechat, T., Gajewski, A., Korbei, B., Gerlich, D., Daigle, N., Haraguchi, T., Furukawa, K., Ellenberg, J. and Foisner, R. (2004). LAP2alpha and BAF transiently localize to telomeres and specific regions on chromatin during nuclear assembly. *J Cell Sci* **117**, 6117-28.

Dorner, D., Vlcek, S., Foeger, N., Gajewski, A., Makolm, C., Gotzmann, J., Hutchison, C. J. and Foisner, R. (2006). Lamina-associated polypeptide 2alpha regulates cell cycle progression and differentiation via the retinoblastoma-E2F pathway. *J Cell Biol* **173**, 83-93.

Dunin-Horkawicz, S., Feder, M. and Bujnicki, J. M. (2006). Phylogenomic analysis of the GIY-YIG nuclease superfamily. *BMC Genomics* **7**, 98.

Ellis, J. A., Craxton, M., Yates, J. R. and Kendrick-Jones, J. (1998). Aberrant intracellular targeting and cell cycle-dependent phosphorylation of emerin contribute to the Emery-Dreifuss muscular dystrophy phenotype. *J Cell Sci* **111 (Pt 6)**, 781-92.

Foisner, R. (2001). Inner nuclear membrane proteins and the nuclear lamina. *J Cell Sci* **114**, 3791-2.

Forne, I., Carrascal, M., Martinez-Lostao, L., Abian, J., Rodriguez-Sanchez, J. L. and Juarez, C. (2003). Identification of the autoantigen HB as the barrier-to-autointegration factor. *J Biol Chem* **278**, 50641-4.

Furukawa, K. (1999). LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction. *J Cell Sci* **112 (Pt 15)**, 2485-92.

Gerner, C., Gotzmann, J., Frohwein, U., Schamberger, C., Ellinger, A. and Sauermann, G. (2002). Proteome analysis of nuclear matrix proteins during apoptotic chromatin condensation. *Cell Death Differ* **9**, 671-81.

Gruenbaum, Y., Margalit, A., Goldman, R. D., Shumaker, D. K. and Wilson, K. L. (2005). The nuclear lamina comes of age. *Nat Rev Mol Cell Biol* **6**, 21-31.

Haraguchi, T., Holaska, J. M., Yamane, M., Koujin, T., Hashiguchi, N., Mori, C., Wilson, K. L. and Hiraoka, Y. (2004). Emerin binding to Btf, a death-promoting transcriptional repressor, is disrupted by a missense mutation that causes Emery-Dreifuss muscular dystrophy. *Eur J Biochem* **271**, 1035-45.

Haraguchi, T., Koujin, T., Segura-Totten, M., Lee, K. K., Matsuoka, Y., Yoneda, Y., Wilson, K. L. and Hiraoka, Y. (2001). BAF is required for emerin assembly into the reforming nuclear envelope. *J Cell Sci* **114**, 4575-85.

Hellemans, J., Preobrazhenska, O., Willaert, A., Debeer, P., Verdonk, P. C., Costa, T., Janssens, K., Menten, B., Van Roy, N., Vermeulen, S. J. et al. (2004). Loss-of-function mutations in LEMD3 result in osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis. *Nat Genet* **36**, 1213-8.

Herrmann, G., Kais, S., Hoffbauer, J., Shah-Hosseini, K., Bruggenolte, N., Schober, H., Fasi, M. and Schar, P. (2007). Conserved interactions of the splicing factor Ntr1/Spp382 with proteins involved in DNA double-strand break repair and telomere metabolism. *Nucleic Acids Res* **35**, 2321-32.

Holaska, J. M., Lee, K. K., Kowalski, A. K. and Wilson, K. L. (2003). Transcriptional repressor germ cell-less (GCL) and barrier to autointegration factor (BAF) compete for binding to emerin in vitro. *J Biol Chem* **278**, 6969-75.

Holaska, J. M., Rais-Bahrami, S. and Wilson, K. L. (2006). Lmo7 is an emerin-binding protein that regulates the transcription of emerin and many other muscle-relevant genes. *Hum Mol Genet* **15**, 3459-72.

Jacque, J. M. and Stevenson, M. (2006). The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. *Nature* **441**, 641-5.

Jayasinghe, S., Hristova, K. and White, S. H. (2001). MPtopo: A database of membrane protein topology. *Protein Sci* **10**, 455-8.

Krogh, A., Larsson, B., von Heijne, G. and Sonnhammer, E. L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* **305**, 567-80.

Kutay, U. and Guttinger, S. (2005). Leucine-rich nuclear-export signals: born to be weak. *Trends Cell Biol* **15**, 121-4.

Laguri, C., Gilquin, B., Wolff, N., Romi-Lebrun, R., Courchay, K., Callebaut, I., Worman, H. J. and Zinn-Justin, S. (2001). Structural characterization of the LEM motif common to three human inner nuclear membrane proteins. *Structure* **9**, 503-11.

Lee, K. K., Gruenbaum, Y., Spann, P., Liu, J. and Wilson, K. L. (2000). C. elegans nuclear envelope proteins emerin, MAN1, lamin, and nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis. *Mol Biol Cell* **11**, 3089-99.

Lee, K. K., Haraguchi, T., Lee, R. S., Koujin, T., Hiraoka, Y. and Wilson, K. L. (2001). Distinct functional domains in emerin bind lamin A and DNA-bridging protein BAF. *J Cell Sci* **114**, 4567-73.

Lee, K. K. and Wilson, K. L. (2004). All in the family: evidence for four new LEM-domain proteins Lem2 (NET-25), Lem3, Lem4 and Lem5 in the human genome. *Symp Soc Exp Biol*, 329-39.

Letunic, I., Copley, R. R., Schmidt, S., Ciccarelli, F. D., Doerks, T., Schultz, J., Ponting, C. P. and Bork, P. (2004). SMART 4.0: towards genomic data integration. *Nucleic Acids Res* **32**, D142-4.

Lin, F., Blake, D. L., Callebaut, I., Skerjanc, I. S., Holmer, L., McBurney, M. W., Paulin-Levasseur, M. and Worman, H. J. (2000). MAN1, an inner nuclear membrane protein

that shares the LEM domain with lamina-associated polypeptide 2 and emerin. *J Biol Chem* **275**, 4840-7.

Liu, J., Lee, K. K., Segura-Totten, M., Neufeld, E., Wilson, K. L. and Gruenbaum, Y. (2003). MAN1 and emerin have overlapping function(s) essential for chromosome segregation and cell division in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **100**, 4598-603.

Mansharamani, M. and Wilson, K. L. (2005). Direct binding of nuclear membrane protein MAN1 to emerin in vitro and two modes of binding to barrier-to-autointegration factor. *J Biol Chem* **280**, 13863-70.

Margalit, A., Brachner, A., Gotzmann, J., Foisner, R. and Gruenbaum, Y. (2007a). Barrier-to-autointegration factor--a BAFfling little protein. *Trends Cell Biol* **17**, 202-8.

Margalit, A., Neufeld, E., Feinstein, N., Wilson, K. L., Podbilewicz, B. and Gruenbaum, Y. (2007b). Barrier to autointegration factor blocks premature cell fusion and maintains adult muscle integrity in C. elegans. *J Cell Biol* **178**, 661-73.

Margalit, A., Segura-Totten, M., Gruenbaum, Y. and Wilson, K. L. (2005a). Barrier-to-autointegration factor is required to segregate and enclose chromosomes within the nuclear envelope and assemble the nuclear lamina. *Proc Natl Acad Sci U S A* **102**, 3290-5.

Margalit, A., Vlcek, S., Gruenbaum, Y. and Foisner, R. (2005b). Breaking and making of the nuclear envelope. *J Cell Biochem* **95**, 454-65.

Markiewicz, E., Tilgner, K., Barker, N., van de Wetering, M., Clevers, H., Dorobek, M., Hausmanowa-Petrusewicz, I., Ramaekers, F. C., Broers, J. L., Blankesteijn, W. M. et al. (2006). The inner nuclear membrane protein emerin regulates beta-catenin activity by restricting its accumulation in the nucleus. *Embo J* **25**, 3275-85.

Melcon, G., Kozlov, S., Cutler, D. A., Sullivan, T., Hernandez, L., Zhao, P., Mitchell, S., Nader, G., Bakay, M., Rottman, J. N. et al. (2006). Loss of emerin at the nuclear envelope disrupts the Rb1/E2F and MyoD pathways during muscle regeneration. *Hum Mol Genet* **15**, 637-51.

Mosavi, L. K., Cammett, T. J., Desrosiers, D. C. and Peng, Z. Y. (2004). The ankyrin repeat as molecular architecture for protein recognition. *Protein Sci* **13**, 1435-48.

Osada, S., Ohmori, S. Y. and Taira, M. (2003). XMAN1, an inner nuclear membrane protein, antagonizes BMP signaling by interacting with Smad1 in Xenopus embryos. *Development* **130**, 1783-94.

Padan, R., Nainudel-Epszteyn, S., Goitein, R., Fainsod, A. and Gruenbaum, Y. (1990). Isolation and characterization of the Drosophila nuclear envelope otefin cDNA. *J Biol Chem* **265**, 7808-13.

Pan, D., Estevez-Salmeron, L. D., Stroschein, S. L., Zhu, X., He, J., Zhou, S. and Luo, K. (2005). The integral inner nuclear membrane protein MAN1 physically interacts with the R-Smad proteins to repress signaling by the transforming growth factor-{beta} superfamily of cytokines. *J Biol Chem* **280**, 15992-6001.

Prunuske, A. J. and Ullman, K. S. (2006). The nuclear envelope: form and reformation. *Curr Opin Cell Biol* **18**, 108-16.

Schirmer, E. C. and Foisner, R. (2007). Proteins that associate with lamins: many faces, many functions. *Exp Cell Res* **313**, 2167-79.

Schmitz, C., Kinge, P. and Hutter, H. (2007). Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive Caenorhabditis elegans strain nre-1(hd20) lin-15b(hd126). *Proc Natl Acad Sci U S A* **104**, 834-9.

Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proc Natl Acad Sci U S A* **95**, 5857-64.

Sedgwick, S. G. and Smerdon, S. J. (1999). The ankyrin repeat: a diversity of interactions on a common structural framework. *Trends Biochem Sci* **24**, 311-6.

Shimi, T., Koujin, T., Segura-Totten, M., Wilson, K. L., Haraguchi, T. and Hiraoka, Y. (2004). Dynamic interaction between BAF and emerin revealed by FRAP, FLIP, and FRET analyses in living HeLa cells. *J Struct Biol* **147**, 31-41.

Shumaker, D. K., Lee, K. K., Tanhehco, Y. C., Craigie, R. and Wilson, K. L. (2001). LAP2 binds to BAF.DNA complexes: requirement for the LEM domain and modulation by variable regions. *Embo J* **20**, 1754-64.

Somech, R., Gal-Yam, E. N., Shaklai, S., Geller, O., Amariglio, N., Rechavi, G. and Simon, A. J. (2007). Enhanced expression of the nuclear envelope LAP2 transcriptional repressors in normal and malignant activated lymphocytes. *Ann Hematol* **86**, 393-401.

Spalding, J. B. and Lammers, P. J. (2004). BLAST Filter and GraphAlign: rule-based formation and analysis of sets of related DNA and protein sequences. *Nucleic Acids Res* **32**, W26-32.

Suzuki, Y., Yang, H. and Craigie, R. (2004). LAP2alpha and BAF collaborate to organize the Moloney murine leukemia virus preintegration complex. *Embo J* **23**, 4670-8.

Theodor, L., Shoham, J., Berger, R., Gokkel, E., Trachtenbrot, L., Simon, A. J., Brok-Simon, F., Nir, U., Ilan, E., Zevin-Sonkin, D. et al. (1997). Ubiquitous expression of a cloned murine thymopoietin cDNA. *Acta Haematol* **97**, 153-63.

Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-80.

Ulbert, S., Antonin, W., Platani, M. and Mattaj, I. W. (2006). The inner nuclear membrane protein Lem2 is critical for normal nuclear envelope morphology. *FEBS Lett* **580**, 6435-41.

Van Maele, B., Busschots, K., Vandekerckhove, L., Christ, F. and Debyser, Z. (2006). Cellular co-factors of HIV-1 integration. *Trends Biochem Sci* **31**, 98-105.

Van Roey, P., Meehan, L., Kowalski, J. C., Belfort, M. and Derbyshire, V. (2002). Catalytic domain structure and hypothesis for function of GIY-YIG intron endonuclease I-TevI. *Nat Struct Biol* **9**, 806-11.

Vlcek, S. and Foisner, R. (2007). A-type lamin networks in light of laminopathic diseases. *Biochim Biophys Acta* **1773**, 661-74.

Vlcek, S., Korbei, B. and Foisner, R. (2002). Distinct functions of the unique C terminus of LAP2alpha in cell proliferation and nuclear assembly. *J Biol Chem* **277**, 18898-907.

Wagner, N. and Krohne, G. (2007). LEM-Domain proteins: new insights into lamin-interacting proteins. *Int Rev Cytol* **261**, 1-46.

Wagner, N., Schmitt, J. and Krohne, G. (2004). Two novel LEM-domain proteins are splice products of the annotated Drosophila melanogaster gene CG9424 (Bocksbeutel). *Eur J Cell Biol* **82**, 605-16.

Wilkinson, F. L., Holaska, J. M., Zhang, Z., Sharma, A., Manilal, S., Holt, I., Stamm, S., Wilson, K. L. and Morris, G. E. (2003). Emerin interacts in vitro with the splicing-associated factor, YT521-B. *Eur J Biochem* **270**, 2459-66.

Yuki, D., Lin, Y. M., Fujii, Y., Nakamura, Y. and Furukawa, Y. (2004). Isolation of LEM domain-containing 1, a novel testis-specific gene expressed in colorectal cancers. *Oncol Rep* **12**, 275-80.

Zheng, R., Ghirlando, R., Lee, M. S., Mizuuchi, K., Krause, M. and Craigie, R. (2000). Barrier-to-autointegration factor (BAF) bridges DNA in a discrete, higher-order nucleoprotein complex. *Proc Natl Acad Sci U S A* **97**, 8997-9002.

3.3.2 LEM3, a novel Player in DNA Recombination/Damage Response?

Damage of genomic DNA, which occurs during replication, after exposure to reactive chemicals or high-energy irradiation, represents a serious threat to individual cells. For unicellular species, it is essential to repair most of the occurring genomic mutations to maintain the genetic information that defines its characteristics. Multicellular organisms not only need to maintain their genomic identity, but mutations also pose a serious threat for the whole organism, as genetic alterations may ultimately lead to deregulated cell proliferation and cancer (Wahl and Carr 2001). Therefore mechanisms to detect and repair damaged DNA developed very early in evolution and are found in all organisms existing today (Aravind *et al.* 1999). The cellular DNA repair machinery has to deal with two main types of DNA damages: (1) Single strand DNA lesions that include nucleotide mismatches, single strand DNA breaks, DNA adducts and thymidine dimers and (2) DNA double strand breaks (Essers *et al.* 2006; Li and Zou 2005). According to the type of DNA damage specific repair pathways are activated: Nucleotide- and base excision repair proteins remove single strand lesions whereas double strand breaks are mostly repaired by the homologous or non-homologous end joining machinery (Wood *et al.* 2005). An early sensor of DNA double strand breaks in eukaryotes is a minor variant of Histone H2A, termed Histone H2A.X (H2A.X) (Rogakou et al. 1998). After DNA lesion H2A.X is instantly phosphorylated by the kinases ATM (Ataxia-Telangeiectasia Mutated gene) (Burma *et al.* 2001), ATR (Ataxia-Telangeiectasia and Rad3-related gene) (Ward and Chen 2001) or DNA-PK (DNA-activated Protein Kinase) (Stiff *et al.* 2004) depending on the primary cause of insult. The phosphorylation of H2A.X (abbreviated as γH2A.X) leads to the formation of DNA-damage foci and consequently triggers several pathways leading to activation of DNA-repair, cell cycle arrest or apoptosis (Rogakou *et al.* 1998).

Higher metazoan organisms not only have to cope with DNA lesions arising “accidentally”, but also insert under distinct circumstances double strand breaks in the genome on purpose. The best investigated and understood situation for such specifically inserted DNA damages are the somatic recombination events during the differentiation of B-and T-cells. In fact, the site-specific V(D)J recombination in B-and T-cells is crucial for a functional, adaptive immune system to defeat pathogens. On the other hand, recombination processes include a serious risk for the whole organism in case of uncontrolled genomic rearrangements which eventually can generate tumorigenic cells (Marculescu *et al.* 2002). Surprisingly, immunocompetent cells are not the only ones undergoing genomic recombination in higher metazoan species. Murine and zebrafish neurons also express proteins known to be involved in somatic recombination in lymphoid cells,

such as RAG1 (Recombination Activated Gene 1) (Chun *et al.* 1991; Feng *et al.* 2005; Jessen *et al.* 2001; Matsuoka *et al.* 1991).

Not unexpectedly, many factors that are involved in DNA damage repair were also found to play a role in recombination, such as proteins of the non-homologous-end-joining repair pathway (Larijani *et al.* 2005). Accordingly, ATM, H2A.X and several other components of the DNA damage response pathway have been reported to be essential in lymphoid cell differentiation. γH2A.X was found in foci at the T-cell receptor α (TCRα) gene in immature T-cells undergoing TCRα recombination (Chen *et al.* 2000) and was found to be necessary for V(D)J- and class switch recombination in differentiating B-cells (Chen *et al.* 2000; Gellert 2002; Reina-San-Martin *et al.* 2003). Furthermore γH2A.X was discovered during male meiosis where it was found to be required for condensation and meiotic pairing of X and Y chromosomes (Fernandez-Capetillo *et al.* 2003; Hunter *et al.* 2001) and additionally it is upregulated upon initiation of DNA fragmentation during apoptosis (Rogakou *et al.* 2000).

We have identified LEM3 as a novel, evolutionary conserved LEM protein expressed in a tissue and cell type restricted manner in mammals. The restricted expression pattern lead us to speculate about a potential role in B-cells (see also chapter 3.3.1) but minor amounts of LEM3 mRNA were also detected in brain, testis and thymus. Furthermore, we found a highly conserved C-terminal region in LEM3 with a predicted GIY-YIG motif (Dunin-Horkawicz *et al.* 2006). As this motif has previously been detected in several proteins with nuclease activity, this may indicate a role of LEM3 in DNA recombination or damage repair. Intriguingly, upon overexpression of LEM3 in cells we found a strong activation of several components known to be involved in DNA damage response, namely ATM, γH2A.X, Chk2 and p53, suggesting that LEM3 either causes DNA damage itself by its predicted nuclease activity or that it may act as a sensor of DNA damage or an activator of the repair response.

Results

Ectopic LEM3 expression causes phosphorylation of ATM, H2A.X, Chk2 and p53

In order to investigate a proposed involvement of LEM3 in DNA-damage response we examined induction of γHistone 2A.X in UV-treated oREC and HeLa cells. At different time points (5, 10, 30, 60 minutes) post irradiation with 50J/m^2 we did not detect a significant difference in the number of γHistone 2A.X positive cells or signal intensity (data not shown) between untransfected control and LEM3-overexpressing cells. However, cells ectopically expressing human or murine LEM3 showed clearly phosphorylated H2A.X without UV treatment (Fig. 38). Interestingly, H2A.X phosphorylation was also significantly affected by the localization of ectopic human LEM3-V5. Expression of mLEM3-V5 in oREC cells, which is predominantly

nuclear, caused a prominent upregulation of γH2A.X as revealed by immunofluorescence (Fig. 38, second row). Human LEM3, in contrast, which is mostly cytoplasmic, only yielded a mild increase in γH2A.X staining in immunofluorescence. However, treatment of hLEM3-V5 expressing cells with the nuclear export inhibitor Leptomycin B, which caused hLEM3 to accumulate in the nucleus (see also chapter 3.3.1), also initiated robust upregulation of the γH2A.X stain, while Leptomycin B had no effect on γH2A.X in hLEM3-lacking untransfected cells. Thus, accumulation of LEM3 in the nucleus causes a dramatic increase in γH2A.X. This effect was specific for LEM3, since overexpression of the inner nuclear membrane protein hLEM2 did not cause upregulation of γH2A.X.

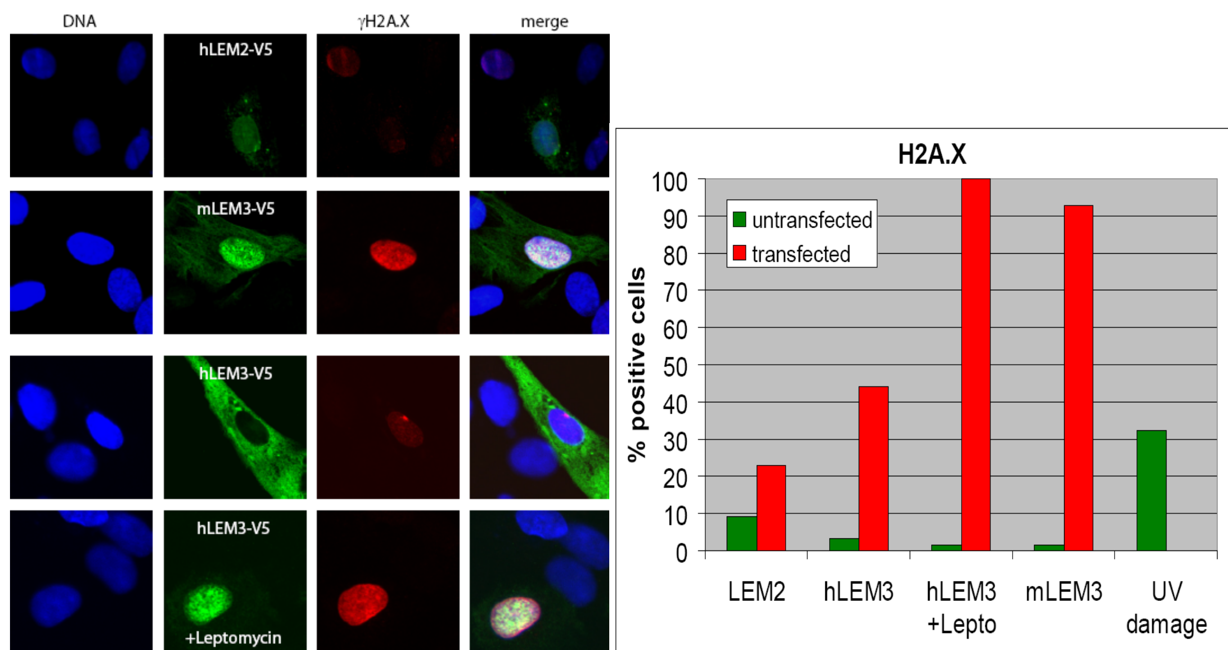


Figure 38: oREC expressing human or murine LEM3-V5 were fixed and stained for V5 (green), γHistone 2A.X (red) and DNA (blue). Human LEM2-V5 served as a negative control for side effects of the transfection procedure. "+Leptomycin" indicates additional treatment with Leptomycin (10 ng/mL) for three hours prior to fixation. Graph: Untransfected and transfected cells on the same petridish were counted for positive γH2A.X staining (n>100 each).

Next, we determined the phosphorylation status of several other components of the DNA damage response pathway in LEM3 expressing cells. We found a striking correlation between the ectopic expression of LEM3 in the nucleus and the phosphorylation of ATM, Chk2 and p53 in MCF7 cells, indicating that the DNA repair pathway is activated in LEM3 expressing cells.

A LEM3 deletion construct lacking the N-terminal Ankyrin repeats as well as the whole C-terminus (LEM3ΔAC) did not cause this effect and served together with untransfected cells as a negative control. Untransfected cells upon UV irradiation (1h post irradiation with $50J/m^2$) served as positive controls (Fig. 39A-D). As MCF7 cells are deficient for Caspase 3 (Kagawa *et*

al. 2001) γH2A.X is not observed upon induction of apoptosis (Rogakou *et al.* 2000). Therefore, we conclude that the observed activation of DNA damage repair proteins upon LEM3 expression is not triggered by an induction of apoptosis.

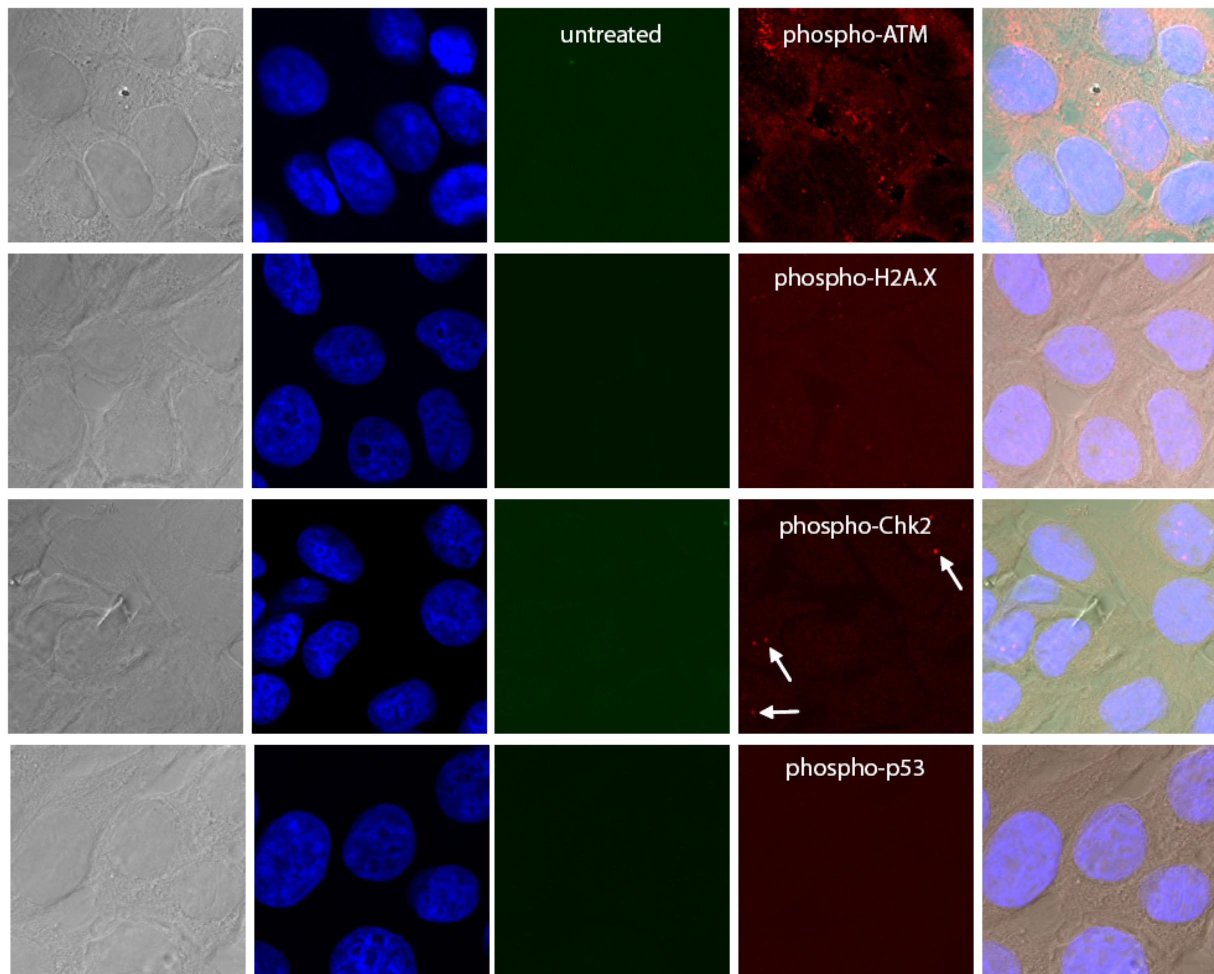


Figure 39A: For negative control, parallel cultures of untreated MCF7 cells were fixed and stained for phosphorylated proteins involved in DNA damage response (red). DNA was visualized by DAPI staining (blue). Arrows indicate localization of phosphorylated Chk2 at centrosomes as described previously (Tsvetkov et al. 2003).

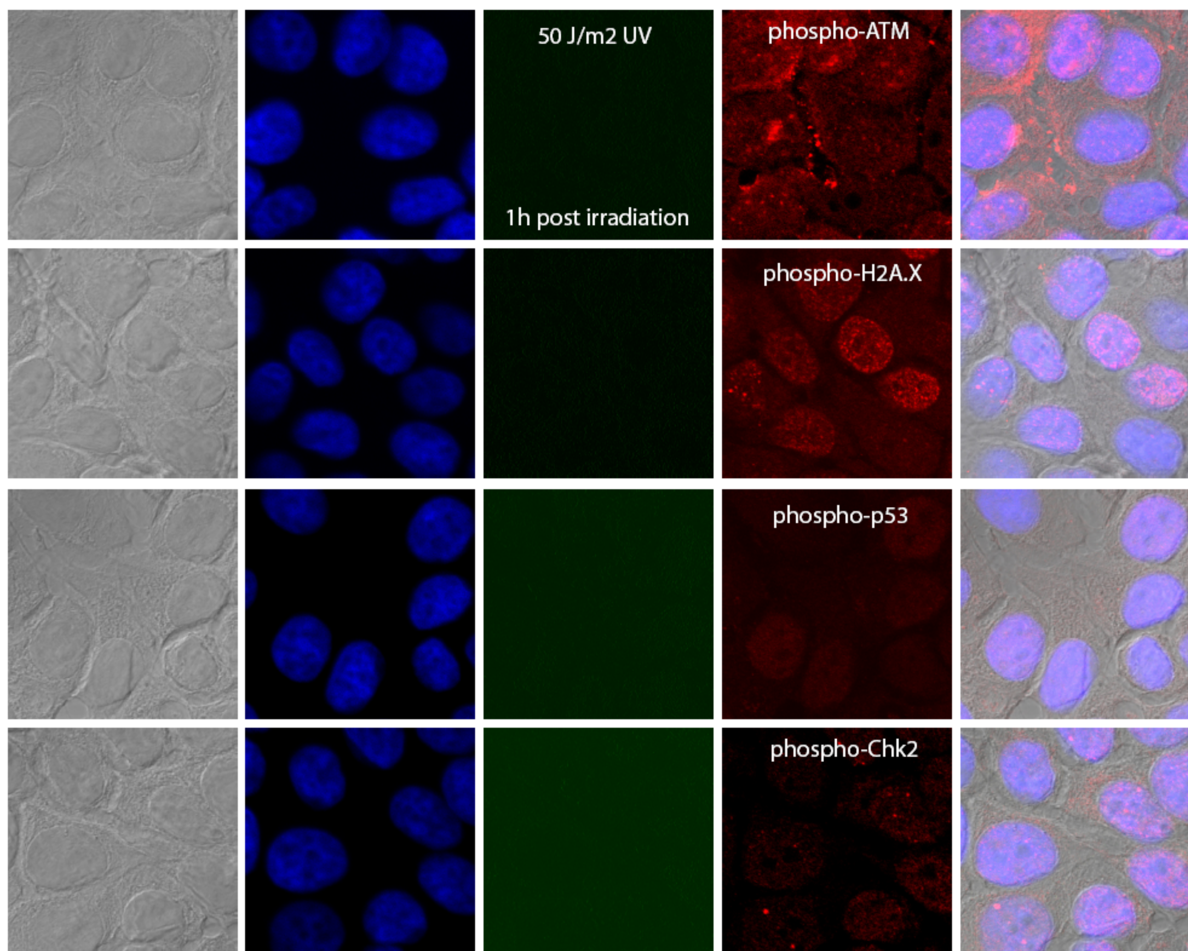


Figure 39B: UV-treated MCF7 cells were fixed and stained for phosphorylated proteins involved in DNA damage response (red) one hour post irradiation with 50J/m^2. DNA staining (blue).

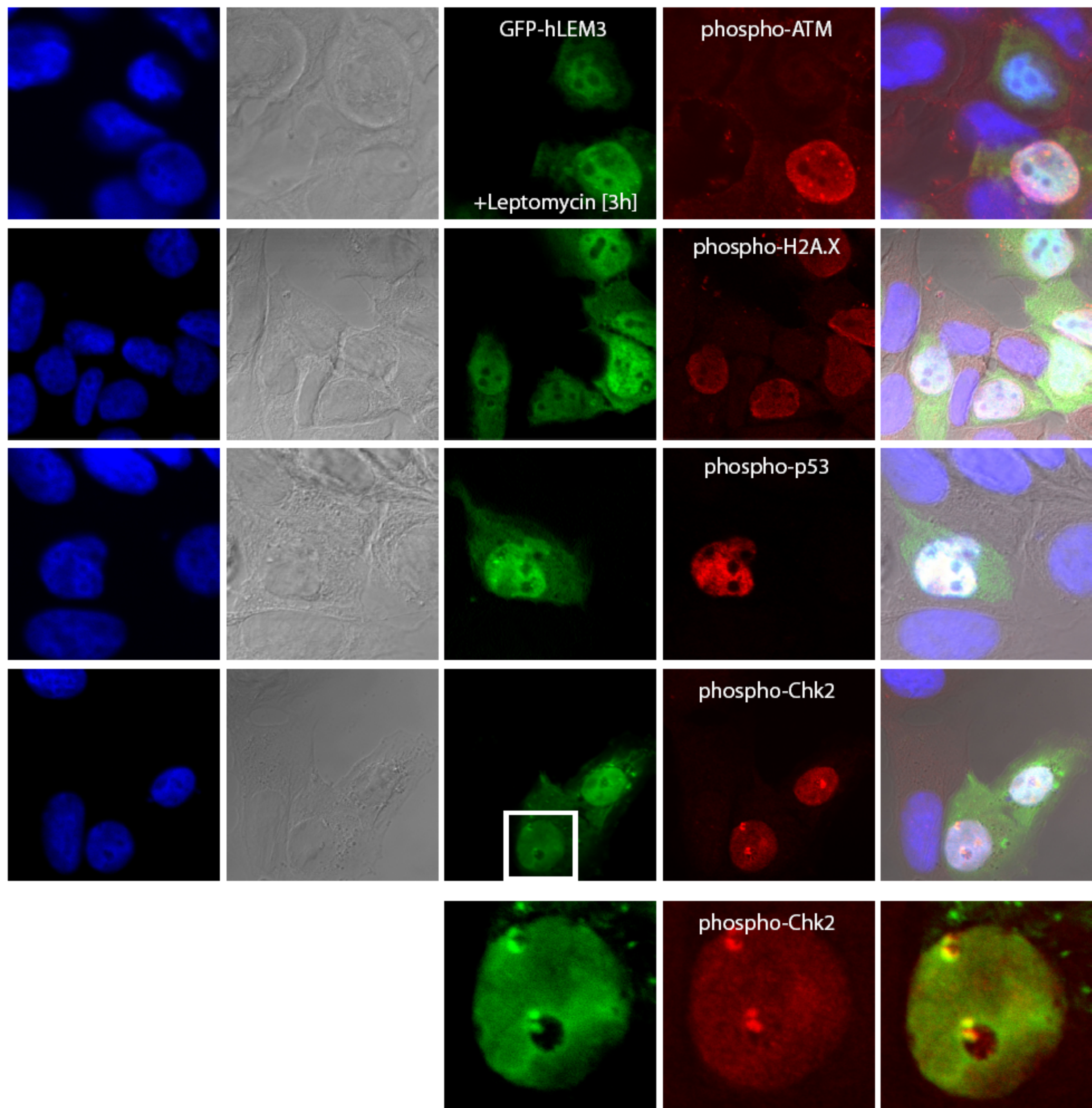


Figure 39C: MCF7 cells were transfected with a GFP-hLEM3α construct. 24h post transfection cells were treated with Leptomycin B for 3h. After Leptomycin treatment, they were fixed and stained for anti-phospho-ATM, anti-γH2A.X, anti-phospho-p53 and anti-phospho-Chk2 (red) DNA was stained with DAPI (blue). Marked cell in forth row ("phospho-Chk2") is shown at higher magnification in the panel below.

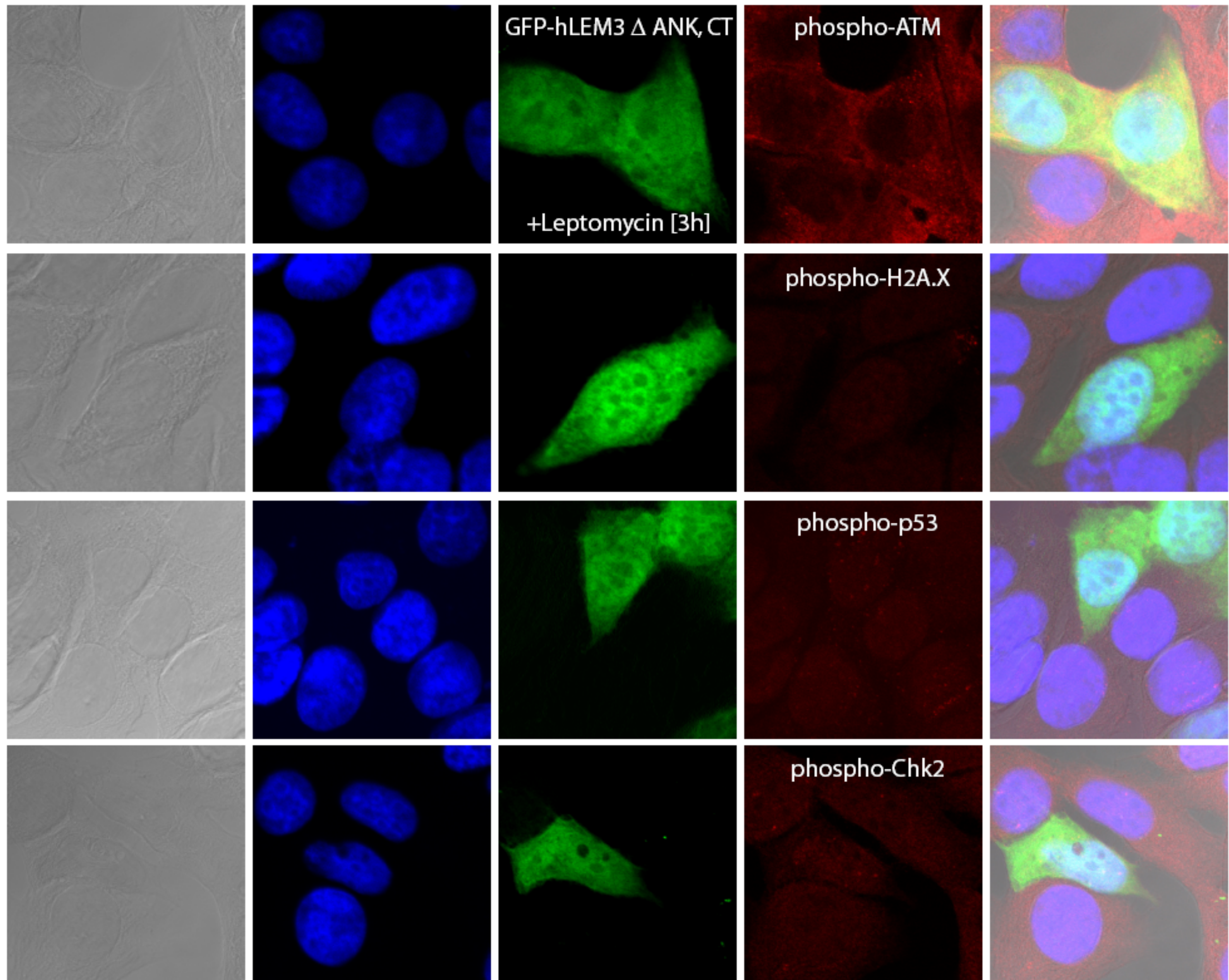


Figure 39D: MCF7 cells were transfected with a GFP-hLEM3ΔANK,CT deletion construct. 24h post transfection cells were treated with Leptomycin for 3h and afterwards fixed and stained for anti-phospho-ATM, anti-γH2A.X, anti-phospho-p53 and anti-phospho-Chk2 (red). DNA was stained with DAPI (blue).

LEM3 might undergo post-translation modification within the nucleus

Western blot analysis of cytoplasmic and nuclear fractions of cells transiently expressing mouse LEM3-V5 confirmed our previous observations in immunofluorescence microscopy showing that mLEM3 is found in the nucleus and cytoplasmic fraction and that it accumulates further in the nuclear fraction upon Leptomycin treatment. (Fig. 40, left blot, mLEM3). Intriguingly, the molecular weight of cytoplasmic versus nuclear mLEM3 differed significantly, revealing a higher molecular weight on SDS gels for nuclear LEM3 as compared to the cytoplasmic pool. This may point to a specific modification, probably a phosphorylation, of LEM3 specifically in the nucleus. Also immunoprecipitated human and murine LEM3-V5 ran faster on SDS gels than LEM3 in lysate inputs (Fig. 40, right blot), which may be caused by incomplete inhibition of phosphatases, and thus dephosphorylation of LEM3 during the precipitation procedure.

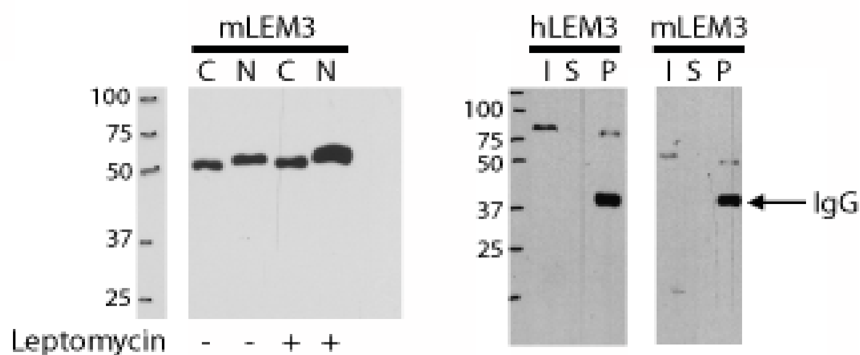


Figure 40: Cells expressing mouse LEM3-V5 were applied to a crude subcellular fractionation procedure and cytoplasmic and nuclear enriched fractions were subjected to SDS-PAGE and immunoblotting (left blot) or V5-immunoprecipitation (right blot). Blots were probed with anti-V5 antibodies. Cytoplasmic and nuclear fractions (C, N), immunoprecipitation input (I), supernatant (S) and precipitated protein (P). "IgG" indicates cross-reactive IgG light chain.

Summary & Conclusions

Genomic recombination and DNA damage response are two intimately linked processes. Multicellular organisms require an adaptive immune system in order to fight efficiently against vital threats posed by pathogens, harmful chemical compounds or malignant cells, which requires genetic rearrangements to create flexibility. The insertion of DNA breaks as needed for genetic rearrangements represents a high risk for the organism, as genomic aberrations can generate transformed cells, eventually leading to cancer. Indeed, a well-characterized reciprocal translocation between the BCR gene locus and the Abl-kinase, thereby forming the so-called "Philadelphia" chromosome, leads to a detrimental fusion product termed BCR/Abl. The dominant BCR/Abl fusion product is the causative factor for almost all cases of chronic

myelogenous leukemia as well as for a high percentage of acute lymphoblastic- and other forms of leukemia (Butturini *et al.* 1996; Salesse and Verfaillie 2002). Other examples for frequently observed aberrant genomic rearrangements in leukemia which cause a misexpression of proto-oncogenes involve bcl-2, an inhibitor of apoptosis, and the transcription factor c-myc (Korsmeyer 1992).

Intriguing and so-far unclear are several studies reporting expression of RAG1 in neuronal samples, which forms together with RAG2 the recombinase responsible for V(D)J and T-cell receptor recombination in lymphoid cells (Fugmann 2001). Based on this, it was speculated that both immunogenic and neurogenic cell differentiation pathways use genetic rearrangements in order to create the enormous heterogeneity inherent to both systems (Chun and Schatz 1999). Interesting in this context is, that LEM3 was also detected at low levels in brain tissue (see also chapter 3.3.1) and was found to be highly expressed in murine sympathetic ganglia (NCBI Unigene database, http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). Two recent screens performed in *C.elegans* mentioned *ce-lem-3* to be involved in axon guidance and to be expressed in motor neurons, respectively (Fox *et al.* 2005; Schmitz *et al.* 2007).

Furthermore, recombination events take place at high frequency in meiosis during maturation of germ cells (about 100-1000 fold more often than during mitosis) (van Heemst and Heyting 2000). Intriguingly, this coincides also with elevated LEM3 expression in testis tissue (see also chapter 3.3.1).

Therefore, we speculate about a role of LEM3 in DNA recombination and/or the subsequent repair procedure. Our working hypothesis is further corroborated by the finding that LEM3 contains a predicted GIY-YIG motif (Dunin-Horkawicz *et al.* 2006), which was previously identified to be linked to nuclease activity in bacterial and phage proteins.

Ectopic expression of human or murine LEM3 resulted in a strong activation of the ATM-dependent signaling pathway. Consistent with our previous notion that human LEM3 represents a nucleo-cytoplasmic shuttling protein (see also chapter 3.3.1) this effect was less pronounced in untreated cells when hLEM3 resides primarily in the cytoplasm. We suggest that the low levels of γH2A.X detected in untreated hLEM3 expressing cells result from minimal amounts of hLEM3 in the nucleus not detectable at steady state in fixed specimen. Whether hLEM3 triggers H2A.X phosphorylation by itself, for instance by causing DNA damage, which would result in turn in phosphorylation of H2A.X and activation of the DNA damage response cascade, or by an indirect way is currently under investigation. According to data obtained in experiments using MCF7 cells, which were shown to lack induction of γH2A.X upon chemically induced apoptosis due to a lack of Caspase-3 activity (Kurokawa *et al.* 1999; Rogakou *et al.* 2000), we conclude that the observed H2A.X phosphorylation is not a consequence of LEM3-provoked apoptosis.

Interestingly, we also noticed a partial co-localization of LEM3 and phosphorylated Chk2 at nuclear speckles, which may indicate a direct or indirect association of these proteins, indicating that LEM3 might also be involved in the DNA repair pathway itself. Noteworthy Chk2 is also found at the centrosomes in non-damaged cells (Tsvetkov *et al.* 2003) and we also noticed partial localization of human and murine LEM3 at centrosomes (data not shown). This finding might become relevant in view of a recently published *in vitro* screen for novel potential chemosensitizers upon treatment of cancer cells with the therapeutic drug Taxol. This screen found LEM3 as one candidate gene for providing protection against Taxol treatment (Whitehurst *et al.* 2007).

If LEM3 indeed functions in recombination, a stringent regulation of (1) expression, (2) localization and (3) activity would be predicted. All three aspects are found for LEM3: (1) expression is limited to certain tissues and alternative splicing may fine-tune full length LEM3 functions; (2) ectopic human LEM3 is strictly excluded from the nucleus by an active Crm-dependent export mechanism, contrary to the localization of the murine ortholog which may be differently regulated; (3) potential posttranslational modification observed for nuclear human and murine LEM3 let us speculate about another level of regulating LEM3 functions. Taken together our findings so far are consistent with a role of LEM3 in recombination being a protein with essential but also potentially harmful functions.

Materials & Methods

Cell culture, transfection procedures and UV irradiation

HeLa and MCF7 cell lines and the embryonic primary rat cells oREC (Cerni *et al.* 1990) were routinely cultivated in DMEM supplemented with 10% fetal calf serum, 2 mM L-Glutamine and 100 U/mL Penicillin/Streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Transient cell transfections were done with Lipofectamine 2000 (Invitrogen) according to the manufacturers recommendations. Induction of apoptosis was done by administration of 1 µM Staurosporine (Sigma) in growth medium for the indicated period. Irradiation of cells with UV was performed using a UV-Stratalinker (Stratagene) set to an energy dose of 50 J/m^2.

Cloning strategy and plasmids

Plasmids pTB-hLEM3 and pTB-mLEM3 are described in chapter 3.3.1, "Material and Methods" section. The vector pTB-hLEM2 containing full length human LEM2 cDNA was described previously (Brachner *et al.* 2005).

Immunofluorescence microscopy & antibodies

Immunofluorescence procedures were performed essentially as described previously (Brachner *et al.* 2005). Briefly, cells were grown on cover slips, washed with PBS once and fixed with ice-cold methanol at -20°C for 2 minutes. After blocking with 0,5% Gelatine/PBS, cells were incubated with primary antibodies, washed 3 times with PBS and probed with fluorescently labeled secondary antibodies. Following three washes with PBS, DNA was stained with DAPI (100 ng/mL) (Sigma) and embedded in MOWIOL (Fluka). Antibodies used in this study were a monoclonal antibody against V5 (Invitrogen) and a mouse anti-γHistone 2A.X purchased from Upstate. All images were taken on a Zeiss LSM-Meta confocal laser scanning microscope (Zeiss), adjusted for brightness and contrast using the LSM Image browser software (Zeiss) and mounted with Photoshop and Illustrator (both Adobe).

Cellular fractionation, immunoprecipitation and Western blotting

Cellular fractionation and Western blotting were performed essentially as described previously (Brachner *et al.* 2005). Immunoprecipitation procedures have been described in chapter 3.3.1, "Materials and Methods" section.

3.3.3 LEM3, Conclusions & Outlook

LEM3 represents a novel type of LEM domain protein, regarding its localization, domain topology and potential functions. LEM proteins have been found so far only within the nucleus, most of them (except for LAP2α) residing within the INM. Emerin, LEM2, MAN1, and LAP2 were shown to interact directly with nuclear Lamins (Brachner *et al.* 2005; Clements *et al.* 2000; Harris *et al.* 1995; Mansharamani and Wilson 2005). It is not known whether LEM3 binds to Lamins too. Tissue restricted expression of LEM3 hints to a role in lymphoid cells. From an evolutionary perspective, LEM3 may have evolved from an ancient protein involved in general DNA damage response, which may have taken over specialized functions associated with recombination- or somatic hypermutation in the differentiation of lymphoid cells. In support of this putative conserved function, a highly conserved motif in the C-terminal part of LEM3 was identified in a computational screen, termed GIY-YIG domain (Dunin-Horkawicz *et al.* 2006). The GIY-YIG motif was previously found to be linked to nuclease functions in bacterial and viral proteins (Dunin-Horkawicz *et al.* 2006; Van Roey *et al.* 2002).

Intriguingly, we observed a strong coincidence between an experimentally induced presence of LEM3 in the nucleus and activation (phosphorylation) of proteins involved in DNA damage response and repair, such as Histone 2A.X, ATM, Chk2 and p53 (chapter 3.3.2). Ectopically expressed human and murine LEM3 co-localized with cytoplasmic Actin stress fibers, γ-Tubulin and nuclear splicing speckles, all of these proteins have been associated with DNA damage response or repair previously (Campalans *et al.* 2007; Herrmann *et al.* 2007; Lesca *et al.* 2005; Okorokov *et al.* 2002).

A comprehensive expression analysis of LEM3 in different phases of B-cell development will be important in future studies to test whether LEM3 expression coincides with a certain differentiation stage. Further on, a knockdown of LEM3 in differentiating pre-B-cells or a knockout mouse model might yield fascinating new insights into the functions of LEM3.

4. References

Adam, S.A. (2001) *The nuclear pore complex*. Genome Biol, 2, REVIEWS0007.

Aebi, U., Cohn, J., Buhle, L. and Gerace, L. (1986) *The nuclear lamina is a meshwork of intermediate-type filaments*. Nature, 323, 560-564.

Akhtar, A. and Gasser, S.M. (2007) *The nuclear envelope and transcriptional control*. Nat Rev Genet, 8, 507-517.

Aravind, L., Walker, D.R. and Koonin, E.V. (1999) *Conserved domains in DNA repair proteins and evolution of repair systems*. Nucleic Acids Res, 27, 1223-1242.

Aravind, L. and Koonin, E.V. (2000) *SAP - a putative DNA-binding motif involved in chromosomal organization*. Trends Biochem Sci, 25, 112-114.

Bakay, M., Wang, Z., Melcon, G., Schiltz, L., Xuan, J., Zhao, P., Sartorelli, V., Seo, J., Pegoraro, E., Angelini, C., Shneiderman, B., Escolar, D., Chen, Y.W., Winokur, S.T., Pachman, L.M., Fan, C., Mandler, R., Nevo, Y., Gordon, E., Zhu, Y., Dong, Y., Wang, Y. and Hoffman, E.P. (2006) *Nuclear envelope dystrophies show a transcriptional fingerprint suggesting disruption of Rb-MyoD pathways in muscle regeneration*. Brain, 129, 996-1013.

Bartova, E., Kozubek, S., Jirsova, P., Kozubek, M., Gajova, H., Lukasova, E., Skalnikova, M., Ganova, A., Koutna, I. and Hausmann, M. (2002) *Nuclear structure and gene activity in human differentiated cells*. J Struct Biol, 139, 76-89.

Beck, M., Lucic, V., Forster, F., Baumeister, W. and Medalia, O. (2007) *Snapshots of nuclear pore complexes in action captured by cryo-electron tomography*. Nature.

Bengtsson, L. (2007) *What MAN1 does to the Smads. TGFbeta/BMP signaling and the nuclear envelope*. Febs J, 274, 1374-1382.

Boguslavsky, R.L., Stewart, C.L. and Worman, H.J. (2006) *Nuclear lamin A inhibits adipocyte differentiation: implications for Dunnigan-type familial partial lipodystrophy*. Hum Mol Genet, 15, 653-663.

Brachner, A., Reipert, S., Foisner, R. and Gotzmann, J. (2005) *LEM2 is a novel MAN1-related inner nuclear membrane protein associated with A-type lamins*. J Cell Sci, 118, 5797-5810.

Bridger, J.M., Foeger, N., Kill, I.R. and Herrmann, H. (2007) *The nuclear lamina. Both a structural framework and a platform for genome organization*. Febs J, 274, 1354-1361.

Broers, J.L., Machiels, B.M., Kuijpers, H.J., Smedts, F., van den Kieboom, R., Raymond, Y. and Ramaekers, F.C. (1997) *A- and B-type lamins are differentially expressed in normal human tissues*. Histochem Cell Biol, 107, 505-517.

Burma, S., Chen, B.P., Murphy, M., Kurimasa, A. and Chen, D.J. (2001) *ATM phosphorylates histone H2AX in response to DNA double-strand breaks*. J Biol Chem, 276, 42462-42467.

Butturini, A., Arlinghaus, R.B. and Gale, R.P. (1996) *BCR/ABL and leukemia*. Leuk Res, 20, 523-529.

Cai, M., Huang, Y., Zheng, R., Wei, S.Q., Ghirlando, R., Lee, M.S., Craigie, R., Gronenborn, A.M. and Clore, G.M. (1998) *Solution structure of the cellular factor BAF responsible for protecting retroviral DNA from autointegration*. Nat Struct Biol, 5, 903-909.

Cai, M., Huang, Y., Ghirlando, R., Wilson, K.L., Craigie, R. and Clore, G.M. (2001) *Solution structure of the constant region of nuclear envelope protein LAP2 reveals two LEM-domain structures: one binds BAF and the other binds DNA*. Embo J, 20, 4399-4407.

Cai, M., Huang, Y., Suh, J.Y., Louis, J.M., Ghirlando, R., Craigie, R. and Clore, G.M. (2007) *Solution NMR structure of the barrier-to-autointegration factor-Emerin complex*. J Biol Chem, 282, 14525-14535.

Campalans, A., Amouroux, R., Bravard, A., Epe, B. and Radicella, J.P. (2007) *UVA irradiation induces relocalisation of the DNA repair protein hOGG1 to nuclear speckles*. J Cell Sci, 120, 23-32.

Caputo, S., Couprie, J., Duband-Goulet, I., Konde, E., Lin, F., Braud, S., Gondry, M., Gilquin, B., Worman, H.J. and Zinn-Justin, S. (2006) *The carboxyl-terminal nucleoplasmic region of MAN1 exhibits a DNA binding winged helix domain*. J Biol Chem, 281, 18208-18215.

Cerni, C., Patocka, K. and Meneguzzi, G. (1990) *Immortalization of primary rat embryo cells by human papillomavirus type 11 DNA is enhanced upon cotransfer of ras*. Virology, 177, 427-436.

Chen, H.T., Bhandoola, A., Difilippantonio, M.J., Zhu, J., Brown, M.J., Tai, X., Rogakou, E.P., Brotz, T.M., Bonner, W.M., Ried, T. and Nussenzweig, A. (2000) *Response to RAG-mediated VDJ cleavage by NBS1 and gamma-H2AX.* Science, 290, 1962-1965.

Chen, I.H., Huber, M., Guan, T., Bubeck, A. and Gerace, L. (2006) *Nuclear envelope transmembrane proteins (NETs) that are up-regulated during myogenesis*. BMC Cell Biol, 7, 38.

Chen, M. and Shen, X. (2007) *Nuclear actin and actin-related proteins in chromatin dynamics*. Curr Opin Cell Biol, 19, 326-330.

Chun, J. and Schatz, D.G. (1999) *Developmental neurobiology: Alternative ends for a familiar story?* Curr Biol, 9, R251-253.

Chun, J.J., Schatz, D.G., Oettinger, M.A., Jaenisch, R. and Baltimore, D. (1991) *The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system*. Cell, 64, 189-200.

Cioce, M. and Lamond, A.I. (2005) *Cajal bodies: a long history of discovery*. Annu Rev Cell Dev Biol, 21, 105-131.
Clements, L., Manilal, S., Love, D.R. and Morris, G.E. (2000) *Direct interaction between emerin and lamin A*. Biochem Biophys Res Commun, 267, 709-714.
Collard, J.F., Senecal, J.L. and Raymond, Y. (1992) *Redistribution of nuclear lamin A is an early event associated with differentiation of human promyelocytic leukemia HL-60 cells*. J Cell Sci, 101 (Pt 3), 657-670.
Constantinescu, D., Gray, H.L., Sammak, P.J., Schatten, G.P. and Csoka, A.B. (2006) *Lamin A/C expression is a marker of mouse and human embryonic stem cell differentiation*. Stem Cells, 24, 177-185.
Crisp, M., Liu, Q., Roux, K., Rattner, J.B., Shanahan, C., Burke, B., Stahl, P.D. and Hodzic, D. (2006) *Coupling of the nucleus and cytoplasm: role of the LINC complex*. J Cell Biol, 172, 41-53.
D'Angelo, M.A. and Hetzer, M.W. (2006) *The role of the nuclear envelope in cellular organization*. Cell Mol Life Sci, 63, 316-332.
de Duve, C. (2007) *The origin of eukaryotes: a reappraisal*. Nat Rev Genet, 8, 395-403.
Dechat, T., Gotzmann, J., Stockinger, A., Harris, C.A., Talle, M.A., Siekierka, J.J. and Foisner, R. (1998) *Detergent-salt resistance of LAP2alpha in interphase nuclei and phosphorylation-dependent association with chromosomes early in nuclear assembly implies functions in nuclear structure dynamics*. Embo J, 17, 4887-4902.
Dechat, T., Shimi, T., Adam, S.A., Rusinol, A.E., Andres, D.A., Spielmann, H.P., Sinensky, M.S. and Goldman, R.D. (2007) *Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging*. Proc Natl Acad Sci U S A, 104, 4955-4960.
Dellaire, G. and Bazett-Jones, D.P. (2004) *PML nuclear bodies: dynamic sensors of DNA damage and cellular stress*. Bioessays, 26, 963-977.
Devos, D., Dokudovskaya, S., Alber, F., Williams, R., Chait, B.T., Sali, A. and Rout, M.P. (2004) *Components of coated vesicles and nuclear pore complexes share a common molecular architecture*. PLoS Biol, 2, e380.
Dorner, D., Vlcek, S., Foeger, N., Gajewski, A., Makolm, C., Gotzmann, J., Hutchison, C.J. and Foisner, R. (2006) *Lamina-associated polypeptide 2alpha regulates cell cycle progression and differentiation via the retinoblastoma-E2F pathway*. J Cell Biol, 173, 83-93.
Dorner, D., Gotzmann, J. and Foisner, R. (2007) *Nucleoplasmic lamins and their interaction partners, LAP2alpha, Rb, and BAF, in transcriptional regulation*. Febs J, 274, 1362-1373.
Dunin-Horkawicz, S., Feder, M. and Bujnicki, J.M. (2006) *Phylogenomic analysis of the GIY-YIG nuclease superfamily*. BMC Genomics, 7, 98.

Dyer, J.A., Kill, I.R., Pugh, G., Quinlan, R.A., Lane, E.B. and Hutchison, C.J. (1997) *Cell cycle changes in A-type lamin associations detected in human dermal fibroblasts using monoclonal antibodies*. Chromosome Res, 5, 383-394.

Enoch, T., Peter, M., Nurse, P. and Nigg, E.A. (1991) *p34cdc2 acts as a lamin kinase in fission yeast*. J Cell Biol, 112, 797-807.

Essers, J., Vermeulen, W. and Houtsmuller, A.B. (2006) *DNA damage repair: anytime, anywhere?* Curr Opin Cell Biol, 18, 240-246.

Feng, B., Bulchand, S., Yaksi, E., Friedrich, R.W. and Jesuthasan, S. (2005) *The recombination activation gene 1 (Rag1) is expressed in a subset of zebrafish olfactory neurons but is not essential for axon targeting or amino acid detection*. BMC Neurosci, 6, 46.

Fernandez-Capetillo, O., Mahadevaiah, S.K., Celeste, A., Romanienko, P.J., Camerini-Otero, R.D., Bonner, W.M., Manova, K., Burgoyne, P. and Nussenzweig, A. (2003) *H2AX is required for chromatin remodeling and inactivation of sex chromosomes in male mouse meiosis*. Dev Cell, 4, 497-508.

Foisner, R. (2001) *Inner nuclear membrane proteins and the nuclear lamina*. J Cell Sci, 114, 3791-3792.

Fong, L.G., Ng, J.K., Lammerding, J., Vickers, T.A., Meta, M., Cote, N., Gavino, B., Qiao, X., Chang, S.Y., Young, S.R., Yang, S.H., Stewart, C.L., Lee, R.T., Bennett, C.F., Bergo, M.O. and Young, S.G. (2006) *Prelamin A and lamin A appear to be dispensable in the nuclear lamina*. J Clin Invest, 116, 743-752.

Forler, D., Rabut, G., Ciccarelli, F.D., Herold, A., Kocher, T., Niggeweg, R., Bork, P., Ellenberg, J. and Izaurralde, E. (2004) *RanBP2/Nup358 provides a major binding site for NXF1-p15 dimers at the nuclear pore complex and functions in nuclear mRNA export*. Mol Cell Biol, 24, 1155-1167.

Foster, H.A. and Bridger, J.M. (2005) *The genome and the nucleus: a marriage made by evolution. Genome organisation and nuclear architecture*. Chromosoma, 114, 212-229.

Fox, R.M., Von Stetina, S.E., Barlow, S.J., Shaffer, C., Olszewski, K.L., Moore, J.H., Dupuy, D., Vidal, M. and Miller, D.M., 3rd (2005) *A gene expression fingerprint of C. elegans embryonic motor neurons*. BMC Genomics, 6, 42.

Fuerst, J.A. (2005) *Intracellular compartmentation in planctomycetes*. Annu Rev Microbiol, 59, 299-328.

Fugmann, S.D. (2001) *RAG1 and RAG2 in V(D)J recombination and transposition*. Immunol Res, 23, 23-39.

Furukawa, K. and Hotta, Y. (1993) *cDNA cloning of a germ cell specific lamin B3 from mouse spermatocytes and analysis of its function by ectopic expression in somatic cells*. Embo J, 12, 97-106.

Furukawa, K., Inagaki, H. and Hotta, Y. (1994) *Identification and cloning of an mRNA coding for a germ cell-specific A-type lamin in mice*. Exp Cell Res, 212, 426-430.

Furukawa, K., Pante, N., Aebi, U. and Gerace, L. (1995) *Cloning of a cDNA for lamina-associated polypeptide 2 (LAP2) and identification of regions that specify targeting to the nuclear envelope*. Embo J, 14, 1626-1636.

Furukawa, K. (1999) *LAP2 binding protein 1 (L2BP1/BAF) is a candidate mediator of LAP2-chromatin interaction*. J Cell Sci, 112 (Pt 15), 2485-2492.

Galcheva-Gargova, Z. and Stateva, L. (1988) *Immunological identification of two lamina-like proteins in Saccharomyces cerevisiae*. Biosci Rep, 8, 287-291.

Gellert, M. (2002) *V(D)J recombination: RAG proteins, repair factors, and regulation*. Annu Rev Biochem, 71, 101-132.

Genovese, C., Trani, D., Caputi, M. and Claudio, P.P. (2006) *Cell cycle control and beyond: emerging roles for the retinoblastoma gene family*. Oncogene, 25, 5201-5209.

Georgatos, S.D., Maroulakou, I. and Blobel, G. (1989) *Lamin A, lamin B, and lamin B receptor analogues in yeast*. J Cell Biol, 108, 2069-2082.

Goodson, H.V. and Hawse, W.F. (2002) *Molecular evolution of the actin family*. J Cell Sci, 115, 2619-2622.

Gotzmann, J. and Foisner, R. (1999) *Lamins and lamin-binding proteins in functional chromatin organization*. Crit Rev Eukaryot Gene Expr, 9, 257-265.

Gotzmann, J. and Foisner, R. (2004) *Lamins and Emerin in muscular dystrophy: the nuclear envelope connection.*, Winder SJ (eds) Molecular mechanisms of muscular dystrophies., pp. pp 1–18.

Gotzmann, J. and Foisner, R. (2006) *A-type lamin complexes and regenerative potential: a step towards understanding laminopathic diseases?* Histochem Cell Biol, 125, 33-41.

Gruenbaum, Y., Goldman, R.D., Meyuhas, R., Mills, E., Margalit, A., Fridkin, A., Dayani, Y., Prokocimer, M. and Enosh, A. (2003) *The nuclear lamina and its functions in the nucleus*. Int Rev Cytol, 226, 1-62.

Guidotti, G. (1972) *Membrane proteins*. Annu Rev Biochem, 41, 731-752.

Halaschek-Wiener, J. and Brooks-Wilson, A. (2007) *Progeria of stem cells: stem cell exhaustion in Hutchinson-Gilford progeria syndrome*. J Gerontol A Biol Sci Med Sci, 62, 3-8.

Haraguchi, T., Koujin, T., Osakada, H., Kojidani, T., Mori, C., Masuda, H. and Hiraoka, Y. (2007) *Nuclear localization of barrier-to-autointegration factor is correlated with progression of S phase in human cells*. J Cell Sci, 120, 1967-1977.

Harris, C.A., Andryuk, P.J., Cline, S.W., Mathew, S., Siekierka, J.J. and Goldstein, G. (1995) *Structure and mapping of the human thymopoietin (TMPO) gene and relationship of human TMPO beta to rat lamin-associated polypeptide 2*. Genomics, 28, 198-205.

Hattier, T., Andrulis, E. and Tartakoff, A.M. (2007) *Immobility, inheritance and plasticity of shape of the yeast nucleus*. BMC Cell Biol, 8, 47.

Hennekam, R.C. (2006) *Hutchinson-Gilford progeria syndrome: review of the phenotype*. Am J Med Genet A, 140, 2603-2624.

Hernandez-Verdun, D. (2006) *The nucleolus: a model for the organization of nuclear functions*. Histochem Cell Biol, 126, 135-148.

Herrmann, G., Kais, S., Hoffbauer, J., Shah-Hosseini, K., Bruggenolte, N., Schober, H., Fasi, M. and Schar, P. (2007) *Conserved interactions of the splicing factor Ntr1/Spp382 with proteins involved in DNA double-strand break repair and telomere metabolism*. Nucleic Acids Res, 35, 2321-2332.

Hetzer, M.W., Walther, T.C. and Mattaj, I.W. (2005) *Pushing the envelope: structure, function, and dynamics of the nuclear periphery*. Annu Rev Cell Dev Biol, 21, 347-380.

Hewett, J.W., Kamm, C., Boston, H., Beauchamp, R., Naismith, T., Ozelius, L., Hanson, P.I., Breakefield, X.O. and Ramesh, V. (2004) *TorsinB--perinuclear location and association with torsinA*. J Neurochem, 89, 1186-1194.

Holaska, J.M. and Wilson, K.L. (2007) *An emerin "proteome": purification of distinct emerin-containing complexes from HeLa cells suggests molecular basis for diverse roles including gene regulation, mRNA splicing, signaling, mechanosensing, and nuclear architecture*. Biochemistry, 46, 8897-8908.

Houben, F., Ramaekers, F.C., Snoeckx, L.H. and Broers, J.L. (2007) *Role of nuclear lamina-cytoskeleton interactions in the maintenance of cellular strength*. Biochim Biophys Acta, 1773, 675-686.

Hunter, N., Borner, G.V., Lichten, M. and Kleckner, N. (2001) *Gamma-H2AX illuminates meiosis*. Nat Genet, 27, 236-238.

Ito, T., Chiba, T., Ozawa, R., Yoshida, M., Hattori, M. and Sakaki, Y. (2001) *A comprehensive two-hybrid analysis to explore the yeast protein interactome*. Proc Natl Acad Sci U S A, 98, 4569-4574.

Ivorra, C., Kubicek, M., Gonzalez, J.M., Sanz-Gonzalez, S.M., Alvarez-Barrientos, A., O'Connor, J.E., Burke, B. and Andres, V. (2006) *A mechanism of AP-1 suppression through interaction of c-Fos with lamin A/C*. Genes Dev, 20, 307-320.

Jessen, J.R., Jessen, T.N., Vogel, S.S. and Lin, S. (2001) *Concurrent expression of recombination activating genes 1 and 2 in zebrafish olfactory sensory neurons*. Genesis, 29, 156-162.

Jockusch, B.M., Schoenenberger, C.A., Stetefeld, J. and Aebi, U. (2006) *Tracking down the different forms of nuclear actin*. Trends Cell Biol, 16, 391-396.

Johnson, B.R., Nitta, R.T., Frock, R.L., Mounkes, L., Barbie, D.A., Stewart, C.L., Harlow, E. and Kennedy, B.K. (2004) *A-type lamins regulate retinoblastoma protein function by promoting subnuclear localization and preventing proteasomal degradation*. Proc Natl Acad Sci U S A, 101, 9677-9682.

Kagawa, S., Gu, J., Honda, T., McDonnell, T.J., Swisher, S.G., Roth, J.A. and Fang, B. (2001) *Deficiency of caspase-3 in MCF7 cells blocks Bax-mediated nuclear fragmentation but not cell death*. Clin Cancer Res, 7, 1474-1480.

King, M.C., Lusk, C.P. and Blobel, G. (2006) *Karyopherin-mediated import of integral inner nuclear membrane proteins*. Nature, 442, 1003-1007.

Korsmeyer, S.J. (1992) *Chromosomal translocations in lymphoid malignancies reveal novel proto-oncogenes*. Annu Rev Immunol, 10, 785-807.

Kudlow, B.A., Kennedy, B.K. and Monnat, R.J., Jr. (2007) *Werner and Hutchinson-Gilford progeria syndromes: mechanistic basis of human progeroid diseases*. Nat Rev Mol Cell Biol, 8, 394-404.

Kuroda, M., Tanabe, H., Yoshida, K., Oikawa, K., Saito, A., Kiyuna, T., Mizusawa, H. and Mukai, K. (2004) *Alteration of chromosome positioning during adipocyte differentiation*. J Cell Sci, 117, 5897-5903.

Kurokawa, H., Nishio, K., Fukumoto, H., Tomonari, A., Suzuki, T. and Saijo, N. (1999) *Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells*. Oncol Rep, 6, 33-37.

Labib, K. and Gambus, A. (2007) *A key role for the GINS complex at DNA replication forks*. Trends Cell Biol, 17, 271-278.

Laguri, C., Gilquin, B., Wolff, N., Romi-Lebrun, R., Courchay, K., Callebaut, I., Worman, H.J. and Zinn-Justin, S. (2001) *Structural characterization of the LEM motif common to three human inner nuclear membrane proteins*. Structure, 9, 503-511.

Lake, J.A. and Rivera, M.C. (1994) *Was the nucleus the first endosymbiont?* Proc Natl Acad Sci U S A, 91, 2880-2881.

Lammerding, J., Hsiao, J., Schulze, P.C., Kozlov, S., Stewart, C.L. and Lee, R.T. (2005) *Abnormal nuclear shape and impaired mechanotransduction in emerin-deficient cells*. J Cell Biol, 170, 781-791.

Lamond, A.I. and Sleeman, J.E. (2003) *Nuclear substructure and dynamics*. Curr Biol, 13, R825-828.

Lamond, A.I. and Spector, D.L. (2003) *Nuclear speckles: a model for nuclear organelles*. Nat Rev Mol Cell Biol, 4, 605-612.

Lanctot, C., Cheutin, T., Cremer, M., Cavalli, G. and Cremer, T. (2007) *Dynamic genome architecture in the nuclear space: regulation of gene expression in three dimensions*. Nat Rev Genet, 8, 104-115.

Lang, B.F., Gray, M.W. and Burger, G. (1999) *Mitochondrial genome evolution and the origin of eukaryotes*. Annu Rev Genet, 33, 351-397.

Larijani, M., Zaheen, A., Frieder, D., Wang, Y., Wu, G.E., Edelmann, W. and Martin, A. (2005) *Lack of MSH2 involvement differentiates V(D)J recombination from other non-homologous end joining events*. Nucleic Acids Res, 33, 6733-6742.

Lee, J.S., Hale, C.M., Panorchan, P., Khatau, S.B., George, J.P., Stewart, C.L., Hodzic, D. and Wirtz, D. (2007) *Effects of nuclear lamin A/C deficiency on cell mechanics, polarization and migration*. Biophys J.

Lee, K.K., Gruenbaum, Y., Spann, P., Liu, J. and Wilson, K.L. (2000) *C. elegans nuclear envelope proteins emerin, MAN1, lamin, and nucleoporins reveal unique timing of nuclear envelope breakdown during mitosis*. Mol Biol Cell, 11, 3089-3099.

Lee, K.K. and Wilson, K.L. (2004) *All in the family: evidence for four new LEM-domain proteins Lem2 (NET-25), Lem3, Lem4 and Lem5 in the human genome*. Symp Soc Exp Biol, 329-339.

Lesca, C., Germanier, M., Raynaud-Messina, B., Pichereaux, C., Etievant, C., Emond, S., Burlet-Schiltz, O., Monsarrat, B., Wright, M. and Defais, M. (2005) *DNA damage induce gamma-tubulin-RAD51 nuclear complexes in mammalian cells*. Oncogene, 24, 5165-5172.

Lewis, A., Felberbaum, R. and Hochstrasser, M. (2007) *A nuclear envelope protein linking nuclear pore basket assembly, SUMO protease regulation, and mRNA surveillance*. J Cell Biol, 178, 813-827.

Li, L. and Zou, L. (2005) *Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells*. J Cell Biochem, 94, 298-306.

Lin, F., Blake, D.L., Callebaut, I., Skerjanc, I.S., Holmer, L., McBurney, M.W., Paulin-Levasseur, M. and Worman, H.J. (2000) *MAN1, an inner nuclear membrane protein that*

shares the LEM domain with lamina-associated polypeptide 2 and emerin. J Biol Chem, 275, 4840-4847.

Liu, J., Lee, K.K., Segura-Totten, M., Neufeld, E., Wilson, K.L. and Gruenbaum, Y. (2003) *MAN1 and emerin have overlapping function(s) essential for chromosome segregation and cell division in Caenorhabditis elegans*. Proc Natl Acad Sci U S A, 100, 4598-4603.

Loewinger, L. and McKeon, F. (1988) *Mutations in the nuclear lamin proteins resulting in their aberrant assembly in the cytoplasm*. Embo J, 7, 2301-2309.

Long, J.Z., Lackan, C.S. and Hadjantonakis, A.K. (2005) *Genetic and spectrally distinct in vivo imaging: embryonic stem cells and mice with widespread expression of a monomeric red fluorescent protein*. BMC Biotechnol, 5, 20.

Lopez-Garcia, P. and Moreira, D. (2006) *Selective forces for the origin of the eukaryotic nucleus*. Bioessays, 28, 525-533.

Lusk, C.P., Blobel, G. and King, M.C. (2007) *Highway to the inner nuclear membrane: rules for the road*. Nat Rev Mol Cell Biol, 8, 414-420.

Machiels, B.M., Zorenc, A.H., Endert, J.M., Kuijpers, H.J., van Eys, G.J., Ramaekers, F.C. and Broers, J.L. (1996) *An alternative splicing product of the lamin A/C gene lacks exon 10*. J Biol Chem, 271, 9249-9253.

Makatsori, D., Kourmouli, N., Polioudaki, H., Shultz, L.D., McLean, K., Theodoropoulos, P.A., Singh, P.B. and Georgatos, S.D. (2004) *The inner nuclear membrane protein lamin B receptor forms distinct microdomains and links epigenetically marked chromatin to the nuclear envelope*. J Biol Chem, 279, 25567-25573.

Manilal, S., Nguyen, T.M., Sewry, C.A. and Morris, G.E. (1996) *The Emery-Dreifuss muscular dystrophy protein, emerin, is a nuclear membrane protein*. Hum Mol Genet, 5, 801-808.

Mans, B.J., Anantharaman, V., Aravind, L. and Koonin, E.V. (2004) *Comparative genomics, evolution and origins of the nuclear envelope and nuclear pore complex*. Cell Cycle, 3, 1612-1637.

Mansharamani, M. and Wilson, K.L. (2005) *Direct binding of nuclear membrane protein MAN1 to emerin in vitro and two modes of binding to barrier-to-autointegration factor*. J Biol Chem, 280, 13863-13870.

Maraldi, N.M., Squarzoni, S., Sabatelli, P., Capanni, C., Mattioli, E., Ognibene, A. and Lattanzi, G. (2005) *Laminopathies: involvement of structural nuclear proteins in the pathogenesis of an increasing number of human diseases*. J Cell Physiol, 203, 319-327.

Maraldi, N.M., Lattanzi, G., Capanni, C., Columbaro, M., Merlini, L., Mattioli, E., Sabatelli, P., Squarzoni, S. and Manzoli, F.A. (2006) *Nuclear envelope proteins and chromatin arrangement: a pathogenic mechanism for laminopathies*. Eur J Histochem, 50, 1-8.

Marculescu, R., Le, T., Simon, P., Jaeger, U. and Nadel, B. (2002) *V(D)J-mediated translocations in lymphoid neoplasms: a functional assessment of genomic instability by cryptic sites*. J Exp Med, 195, 85-98.

Margalit, A., Brachner, A., Gotzmann, J., Foisner, R. and Gruenbaum, Y. (2007a) *Barrier-to-autointegration factor--a BAFfling little protein*. Trends Cell Biol, 17, 202-208.

Margalit, A., Neufeld, E., Feinstein, N., Wilson, K.L., Podbilewicz, B. and Gruenbaum, Y. (2007b) *Barrier to autointegration factor blocks premature cell fusion and maintains adult muscle integrity in C. elegans*. J Cell Biol, 178, 661-673.

Markiewicz, E., Dechat, T., Foisner, R., Quinlan, R.A. and Hutchison, C.J. (2002) *Lamin A/C binding protein LAP2alpha is required for nuclear anchorage of retinoblastoma protein*. Mol Biol Cell, 13, 4401-4413.

Markiewicz, E., Ledran, M. and Hutchison, C.J. (2005) *Remodelling of the nuclear lamina and nucleoskeleton is required for skeletal muscle differentiation in vitro*. J Cell Sci, 118, 409-420.

Markiewicz, E., Tilgner, K., Barker, N., van de Wetering, M., Clevers, H., Dorobek, M., Hausmanowa-Petrusewicz, I., Ramaekers, F.C., Broers, J.L., Blankesteijn, W.M., Salpingidou, G., Wilson, R.G., Ellis, J.A. and Hutchison, C.J. (2006) *The inner nuclear membrane protein emerin regulates beta-catenin activity by restricting its accumulation in the nucleus*. Embo J, 25, 3275-3285.

Martin, W. and Koonin, E.V. (2006) *Introns and the origin of nucleus-cytosol compartmentalization*. Nature, 440, 41-45.

Maske, C.P., Hollinshead, M.S., Higbee, N.C., Bergo, M.O., Young, S.G. and Vaux, D.J. (2003) *A carboxyl-terminal interaction of lamin B1 is dependent on the CAAX endoprotease Rce1 and carboxymethylation*. J Cell Biol, 162, 1223-1232.

Masny, P.S., Bengtsson, U., Chung, S.A., Martin, J.H., van Engelen, B., van der Maarel, S.M. and Winokur, S.T. (2004) *Localization of 4q35.2 to the nuclear periphery: is FSHD a nuclear envelope disease?* Hum Mol Genet, 13, 1857-1871.

Matera, A.G. (1999) *Nuclear bodies: multifaceted subdomains of the interchromatin space*. Trends Cell Biol, 9, 302-309.

Matsuoka, M., Nagawa, F., Okazaki, K., Kingsbury, L., Yoshida, K., Muller, U., Larue, D.T., Winer, J.A. and Sakano, H. (1991) *Detection of somatic DNA recombination in the transgenic mouse brain*. Science, 254, 81-86.

McDonald, D., Carrero, G., Andrin, C., de Vries, G. and Hendzel, M.J. (2006) *Nucleoplasmic beta-actin exists in a dynamic equilibrium between low-mobility polymeric species and rapidly diffusing populations*. J Cell Biol, 172, 541-552.

Minguez, A., Franca, S. and Moreno Diaz de la Espina, S. (1994) *Dinoflagellates have a eukaryotic nuclear matrix with lamin-like proteins and topoisomerase II*. J Cell Sci, 107 (Pt 10), 2861-2873.

Moir, R.D., Spann, T.P., Lopez-Soler, R.I., Yoon, M., Goldman, A.E., Khuon, S. and Goldman, R.D. (2000) *Review: the dynamics of the nuclear lamins during the cell cycle--relationship between structure and function*. J Struct Biol, 129, 324-334.

Montes de Oca, R., Lee, K.K. and Wilson, K.L. (2005) *Binding of barrier to autointegration factor (BAF) to histone H3 and selected linker histones including H1.1*. J Biol Chem, 280, 42252-42262.

Mounkes, L., Kozlov, S., Burke, B. and Stewart, C.L. (2003) *The laminopathies: nuclear structure meets disease*. Curr Opin Genet Dev, 13, 223-230.

Muchir, A. and Worman, H.J. (2007) *Emery-Dreifuss muscular dystrophy*. Curr Neurol Neurosci Rep, 7, 78-83.

Muralikrishna, B., Dhawan, J., Rangaraj, N. and Parnaik, V.K. (2001) *Distinct changes in intranuclear lamin A/C organization during myoblast differentiation*. J Cell Sci, 114, 4001-4011.

Nagano, A. and Arahata, K. (2000) *Nuclear envelope proteins and associated diseases*. Curr Opin Neurol, 13, 533-539.

Nalepa, G. and Harper, J.W. (2004) *Visualization of a highly organized intranuclear network of filaments in living mammalian cells*. Cell Motil Cytoskeleton, 59, 94-108.

Nickerson, J. (2001) *Experimental observations of a nuclear matrix*. J Cell Sci, 114, 463-474.

Okorokov, A.L., Rubbi, C.P., Metcalfe, S. and Milner, J. (2002) *The interaction of p53 with the nuclear matrix is mediated by F-actin and modulated by DNA damage*. Oncogene, 21, 356-367.

Ozaki, T., Saijo, M., Murakami, K., Enomoto, H., Taya, Y. and Sakiyama, S. (1994) *Complex formation between lamin A and the retinoblastoma gene product: identification of the domain on lamin A required for its interaction*. Oncogene, 9, 2649-2653.

Pan, D., Estevez-Salmeron, L.D., Stroschein, S.L., Zhu, X., He, J., Zhou, S. and Luo, K. (2005) *The integral inner nuclear membrane protein MAN1 physically interacts with the R-Smad proteins to repress signaling by the transforming growth factor-{beta} superfamily of cytokines*. J Biol Chem, 280, 15992-16001.

Pederson, T. (2000) *Half a century of "the nuclear matrix"*. Mol Biol Cell, 11, 799-805.

Pederson, T. and Aebi, U. (2005) *Nuclear actin extends, with no contraction in sight*. Mol Biol Cell, 16, 5055-5060.

Pekovic, V., Harborth, J., Broers, J.L., Ramaekers, F.C., van Engelen, B., Lammens, M., von Zglinicki, T., Foisner, R., Hutchison, C. and Markiewicz, E. (2007) *Nucleoplasmic LAP2alpha-lamin A complexes are required to maintain a proliferative state in human fibroblasts*. J Cell Biol, 176, 163-172.

Pennisi, E. (2004) *Evolutionary biology. The birth of the nucleus*. Science, 305, 766-768.

Pestic-Dragovich, L., Stojiljkovic, L., Philimonenko, A.A., Nowak, G., Ke, Y., Settlage, R.E., Shabanowitz, J., Hunt, D.F., Hozak, P. and de Lanerolle, P. (2000) *A myosin I isoform in the nucleus*. Science, 290, 337-341.

Peter, M. and Nigg, E.A. (1991) *Ectopic expression of an A-type lamin does not interfere with differentiation of lamin A-negative embryonal carcinoma cells*. J Cell Sci, 100 (Pt 3), 589-598.

Rankin, J. and Ellard, S. (2006) *The laminopathies: a clinical review*. Clin Genet, 70, 261-274.

Raska, I., Shaw, P.J. and Cmarko, D. (2006) *Structure and function of the nucleolus in the spotlight*. Curr Opin Cell Biol, 18, 325-334.

Reina-San-Martin, B., Difilippantonio, S., Hanitsch, L., Masilamani, R.F., Nussenzweig, A. and Nussenzweig, M.C. (2003) *H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation*. J Exp Med, 197, 1767-1778.

Rober, R.A., Weber, K. and Osborn, M. (1989) *Differential timing of nuclear lamin A/C expression in the various organs of the mouse embryo and the young animal: a developmental study*. Development, 105, 365-378.

Rodriguez-Navarro, S., Igual, J.C. and Perez-Ortin, J.E. (2002) *SRC1: an intron-containing yeast gene involved in sister chromatid segregation*. Yeast, 19, 43-54.

Rogakou, E.P., Pilch, D.R., Orr, A.H., Ivanova, V.S. and Bonner, W.M. (1998) *DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139*. J Biol Chem, 273, 5858-5868.

Rogakou, E.P., Nieves-Neira, W., Boon, C., Pommier, Y. and Bonner, W.M. (2000) *Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139*. J Biol Chem, 275, 9390-9395.

Salesse, S. and Verfaillie, C.M. (2002) *BCR/ABL: from molecular mechanisms of leukemia induction to treatment of chronic myelogenous leukemia*. Oncogene, 21, 8547-8559.

Schirmer, E.C., Florens, L., Guan, T., Yates, J.R., 3rd and Gerace, L. (2003) *Nuclear membrane proteins with potential disease links found by subtractive proteomics*. Science, 301, 1380-1382.

Schirmer, E.C., Florens, L., Guan, T., Yates, J.R., 3rd and Gerace, L. (2005) *Identification of novel integral membrane proteins of the nuclear envelope with potential disease links using subtractive proteomics*. Novartis Found Symp, 264, 63-76; discussion 76-80, 227-230.

Schirmer, E.C. and Gerace, L. (2005) *The nuclear membrane proteome: extending the envelope*. Trends Biochem Sci, 30, 551-558.

Schlame, M., Rua, D. and Greenberg, M.L. (2000) *The biosynthesis and functional role of cardiolipin*. Prog Lipid Res, 39, 257-288.

Schmitz, C., Kinge, P. and Hutter, H. (2007) *Axon guidance genes identified in a large-scale RNAi screen using the RNAi-hypersensitive Caenorhabditis elegans strain nre-1(hd20) lin-15b(hd126)*. Proc Natl Acad Sci U S A, 104, 834-839.

Shumaker, D.K., Lee, K.K., Tanhehco, Y.C., Craigie, R. and Wilson, K.L. (2001) *LAP2 binds to BAF.DNA complexes: requirement for the LEM domain and modulation by variable regions*. Embo J, 20, 1754-1764.

Somech, R., Shaklai, S., Amariglio, N., Rechavi, G. and Simon, A.J. (2005a) *Nuclear envelopathies--raising the nuclear veil*. Pediatr Res, 57, 8R-15R.

Somech, R., Shaklai, S., Geller, O., Amariglio, N., Simon, A.J., Rechavi, G. and Gal-Yam, E.N. (2005b) *The nuclear-envelope protein and transcriptional repressor LAP2beta interacts with HDAC3 at the nuclear periphery, and induces histone H4 deacetylation*. J Cell Sci, 118, 4017-4025.

Spector, D.L. (2001) *Nuclear domains*. J Cell Sci, 114, 2891-2893.

Stewart, C. and Burke, B. (1987) *Teratocarcinoma stem cells and early mouse embryos contain only a single major lamin polypeptide closely resembling lamin B*. Cell, 51, 383-392.

Stiff, T., O'Driscoll, M., Rief, N., Iwabuchi, K., Lobrich, M. and Jeggo, P.A. (2004) *ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation*. Cancer Res, 64, 2390-2396.

Strelkov, S.V., Herrmann, H. and Aebi, U. (2003) *Molecular architecture of intermediate filaments*. Bioessays, 25, 243-251.

Sullivan, T., Escalante-Alcalde, D., Bhatt, H., Anver, M., Bhat, N., Nagashima, K., Stewart, C.L. and Burke, B. (1999) *Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy*. J Cell Biol, 147, 913-920.

Tifft, K.E., Segura-Totten, M., Lee, K.K. and Wilson, K.L. (2006) *Barrier-to-autointegration factor-like (BAF-L): a proposed regulator of BAF*. Exp Cell Res, 312, 478-487.

Tsvetkov, L., Xu, X., Li, J. and Stern, D.F. (2003) *Polo-like kinase 1 and Chk2 interact and co-localize to centrosomes and the midbody*. J Biol Chem, 278, 8468-8475.

Tzur, Y.B., Wilson, K.L. and Gruenbaum, Y. (2006) *SUN-domain proteins: 'Velcro' that links the nucleoskeleton to the cytoskeleton*. Nat Rev Mol Cell Biol, 7, 782-788.

Ulbert, S., Antonin, W., Platani, M. and Mattaj, I.W. (2006) *The inner nuclear membrane protein Lem2 is critical for normal nuclear envelope morphology*. FEBS Lett, 580, 6435-6441.

van Heemst, D. and Heyting, C. (2000) *Sister chromatid cohesion and recombination in meiosis*. Chromosoma, 109, 10-26.

Van Roey, P., Meehan, L., Kowalski, J.C., Belfort, M. and Derbyshire, V. (2002) *Catalytic domain structure and hypothesis for function of GIY-YIG intron endonuclease I-TevI*. Nat Struct Biol, 9, 806-811.

Vergnes, L., Peterfy, M., Bergo, M.O., Young, S.G. and Reue, K. (2004) *Lamin B1 is required for mouse development and nuclear integrity*. Proc Natl Acad Sci U S A, 101, 10428-10433.

Verheijen, R., van Venrooij, W. and Ramaekers, F. (1988) *The nuclear matrix: structure and composition*. J Cell Sci, 90 (Pt 1), 11-36.

Verstraeten, V.L., Broers, J.L., Ramaekers, F.C. and van Steensel, M.A. (2007) *The nuclear envelope, a key structure in cellular integrity and gene expression*. Curr Med Chem, 14, 1231-1248.

Vlcek, S., Dechat, T. and Foisner, R. (2001) *Nuclear envelope and nuclear matrix: interactions and dynamics*. Cell Mol Life Sci, 58, 1758-1765.

Vlcek, S., Korbei, B. and Foisner, R. (2002) *Distinct functions of the unique C terminus of LAP2alpha in cell proliferation and nuclear assembly*. J Biol Chem, 277, 18898-18907.

Vlcek, S. and Foisner, R. (2007) *Lamins and lamin-associated proteins in aging and disease*. Curr Opin Cell Biol, 19, 298-304.

Wagner, N. and Krohne, G. (2007) *LEM-Domain Proteins: New Insights into Lamin-Interacting Proteins*. Int Rev Cytol, 261, 1-46.

Wahl, G.M. and Carr, A.M. (2001) *The evolution of diverse biological responses to DNA damage: insights from yeast and p53*. Nat Cell Biol, 3, E277-286.

Wang, X., Xu, S., Rivolta, C., Li, L.Y., Peng, G.H., Swain, P.K., Sung, C.H., Swaroop, A., Berson, E.L., Dryja, T.P. and Chen, S. (2002) *Barrier to autointegration factor interacts with the cone-rod homeobox and represses its transactivation function*. J Biol Chem, 277, 43288-43300.

Ward, I.M. and Chen, J. (2001) *Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress*. J Biol Chem, 276, 47759-47762.

Whitehurst, A.W., Bodemann, B.O., Cardenas, J., Ferguson, D., Girard, L., Peyton, M., Minna, J.D., Michnoff, C., Hao, W., Roth, M.G., Xie, X.J. and White, M.A. (2007) *Synthetic lethal screen identification of chemosensitizer loci in cancer cells*. Nature, 446, 815-819.

Wilhelmsen, K., Ketema, M., Truong, H. and Sonnenberg, A. (2006) *KASH-domain proteins in nuclear migration, anchorage and other processes*. J Cell Sci, 119, 5021-5029.

Wood, R.D., Mitchell, M. and Lindahl, T. (2005) *Human DNA repair genes, 2005*. Mutat Res, 577, 275-283.

Worman, H.J. and Bonne, G. (2007) *"Laminopathies": A wide spectrum of human diseases*. Exp Cell Res, 313, 2121-2133.

Yuki, D., Lin, Y.M., Fujii, Y., Nakamura, Y. and Furukawa, Y. (2004) *Isolation of LEM domain-containing 1, a novel testis-specific gene expressed in colorectal cancers*. Oncol Rep, 12, 275-280.

5. Appendix

Abbreviations

AP1	Activator Protein 1
ATM	Ataxia-Telangeiectasia Mutated gene
ATR	Ataxia-Telangeiectasia and Rad3-related gene
BAF	Barrier-to-Autointegration Factor
Chk2	Checkpoint protein 2
CT	C-Terminus
DCM	Dilated CardioMyopathy
DNA-PK	DNA-activated Protein Kinase
DMEM	Dulbecco's Modified Eagles Medium
EDMD	Emery-Dreifuss Muscular Dystrophy
ER	Endoplasmic Reticulum
esp	*yeast* Separin
FCS	Fetal Calves Serum
FPLD	Familial Partial Lipo-Dystrophy
GFP	Green Fluorescent Protein
H2A.X	Histone 2A.X
γH2A.X	*phosphorylated* Histone 2A.X
heh	*yeast* helix-extension-helix containing gene
HGPS	Hutchison-Gilford Premature aging Syndrome
INM	Inner Nuclear Membrane
KASH	Klarsicht, ANC1, SYNE1 Homology
LAP2	Lamina-Associated Polypeptide 2
LEM	LAP2, Emerin, MAN1
lmna	*murine* Lamin A/C gene
MAN1	Man AntigeN 1
NE	Nuclear Envelope
NPC	Nuclear Pore Complex
NT	N-Terminus
NUP	NUcleoPorin
ONM	Outer Nuclear Membrane
PAGE	PolyAcrylamide Gel Electrophoresis

PCR	Polymerase Chain Reaction
RAG	Recombination Activated Gene
RFP	Red Fluorescent Protein
RNAi	RNA interference
RT-PCR	Reverse Transcription-PCR
SAP	SAF A/B, Acinus, PIAS
scc	*yeast* Securin
src1	*yeast* Spliced mRNA and cell Cycle-regulated gene
SUN	Sad1-UNC84 homology
V(D)J	Variable (Diversity) Joining

Curriculum Vitae

Name : BRACHNER Andreas

Geburtsdatum : 1. August 1978

Geburtsort : Melk (Niederösterreich)

Nationalität : Österreich

Ausbildung :

1988 - 1996	Neusprachliche AHS und Matura am Stiftsgymnasium Melk.
1997 - 2001	Studium der Humanbiologie an der Universität Wien.
2000	Ein Semester Auslandsstudium an der Universität Oulu (Finnland).
2002 - 2003	Diplomarbeit unter Anleitung von Prof. Christa Cerni (†) am Institut für Krebsforschung der Universität Wien.
Feb. 2003	Sponsion zum Magister der Naturwissenschaften an der Universität Wien.
2004 - 2008	Dissertation im Fach „Molekulare Biologie" unter der Betreuung von Prof. Roland Foisner am Institut für Medizinische Biochemie der Medizinischen Universität Wien.

Publications

Brachner A, Reipert S, Foisner R, Gotzmann J.: *LEM2 is a novel MAN1-related inner nuclear membrane protein associated with A-type lamins.* **J Cell Sci**. 2005 Dec 15;118(Pt 24):5797-810

Brachner A, Sasgary S, Pirker C, Rodgarkia C, Mikula M, Mikulits W, Bergmeister H, Setinek U, Wieser M, Chin SF, Caldas C, Micksche M, Cerni C, Berger W.: *Telomerase- and alternative telomere lengthening-independent telomere stabilization in a metastasis-derived human non-small cell lung cancer cell line: effect of ectopic hTERT.* **Cancer Res**. 2006 Apr 1;66(7):3584-92

Margalit A, Brachner A, Gotzmann J, Foisner R, Gruenbaum Y.: *Barrier-to-autointegration factor--a BAFfling little protein*. **Trends Cell Biol**. 2007 Apr;17(4):202-8