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Lamin B receptor
Multi-tasking at the nuclear envelope

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Key words: lamin B receptor, nuclear envelope, lamins, chromatin, sterol reductase

Abbreviations: LBR, lamin B receptor; NE, nuclear envelope; ONM, outer nuclear membrane; INM, inner nuclear membrane; ER, endoplasmic reticulum; HP1, heterochromatin-associated protein 1; aa, amino acid

Lamin B receptor (LBR) is an integral membrane protein of the interphase nuclear envelope (NE). The N-terminal end resides in the nucleoplasm, binding to lamin B and heterochromatin, with the interactions disrupted during mitosis. The C-terminal end resides within the inner nuclear membrane, retreating with the ER away from condensing chromosomes during mitotic NE breakdown. Some of these properties are interpretable in terms of our current structural knowledge of LBR, but many of the structural features remain unknown. LBR apparently has an evolutionary history which brought together at least two ancient conserved structural domains (i.e., Tudor and sterol reductase). This convergence may have occurred with the emergence of the chordates and echinoderms. It is not clear what survival values have maintained LBR structure during evolution. But it seems likely that roles in post-mitotic nuclear reformation, interphase NE growth and compartmentalization of nuclear architecture might have provided some evolutionary advantage to preservation of the LBR gene.

Introduction

The interphase nuclear envelope (NE) can be regarded as a specialized extension of the endoplasmic reticulum (ER) which functions to compartmentalize and organize the nuclear functions of gene regulation and replication, and provide spatial separation from cytoplasmic functions. Contemporary concepts[1-9] view the NE as a multi-tiered structure with the following components: two parallel membranes [i.e., the outer nuclear membrane (ONM), facing the cytoplasm and studded with ribosomes, and the inner nuclear membrane (INM), facing the nucleoplasm]; a layer of intermediate filament lamins (assumed to stabilize NE structure); a layer of heterochromatin; integral membrane proteins of the INM (assumed to "stitch" the layers together and provide a "platform" for internal nuclear organization). Vertebrate nuclear lamins are encoded by three separate genes for lamins B1, B2 and A (including the alternative splice product, lamin C), all of which possess nuclear localization signals (NLS) in their C-terminal "tail" domains. Prominent among the integral membrane proteins of the INM is lamin B receptor (LBR), which appears to be a chimeric protein with multiple functions during its residence in the NE. Only a few of the estimated ~60–70 NE transmembrane proteins[10] have been characterized. Besides LBR, these include LAP2, emerin, MAN1, SUN1 and 2 and nesprin.[1,6] This review focuses upon the complex structure and function of LBR, summarizing much of the published knowledge and underscoring the numerous gaps in our understanding.

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Discovery and Characterization of Avian LBR

LBR was first described in a publication from the Blobel laboratory in 1988.[11] Lamins A and B were extracted by 8 M urea from turkey erythrocyte nuclear envelope (NE) preparations and labeled with ^{125}Iodine. The labeled lamins were employed in binding assays to nitrocellulose membrane transfers of SDS gel electrophoresis and to suspensions of extracted NE residues. Lamin B highlighted a 58 kD band on the membrane transfers and revealed saturated binding to the NE residues. Lamin A showed substantially less binding. The lamin B binding protein required high KCl/Triton-X-100 to be extracted from NE, and was thus regarded as an integral membrane protein. Furthermore, antibodies directed against lamin B binding protein revealed nuclear rim staining by immunofluorescence microscopy. The authors suggested that the 58 kD protein is the NE "receptor" for lamin B; no receptor was detected for lamin A. In a subsequent study,[12] cDNA plasmids of LBR were isolated from a chicken liver library and a peptide sequence was deduced from the sequenced DNA. Chicken LBR was determined to contain 637 aa. By hydropathy plot analysis, eight putative transmembrane peptide segments were identified within the C-terminal 433 aa. The N-terminal 204 aa was clearly basic (calculated pI ~9.89) and possessed candidate serine phosphorylation sites. In a different study,[13] protein kinase A (PKA) was shown to phosphorylate serine residue(s) of LBR in vivo and in vitro. Furthermore, enzymatic dephosphorylation of the nitrocellulose membranes after protein transfer reduced lamin B binding to the 58 kD LBR band. Evidence was also presented[12] that the C-terminal region is within the inner membrane of the NE and the N-terminal region is in the nucleoplasm, capable of

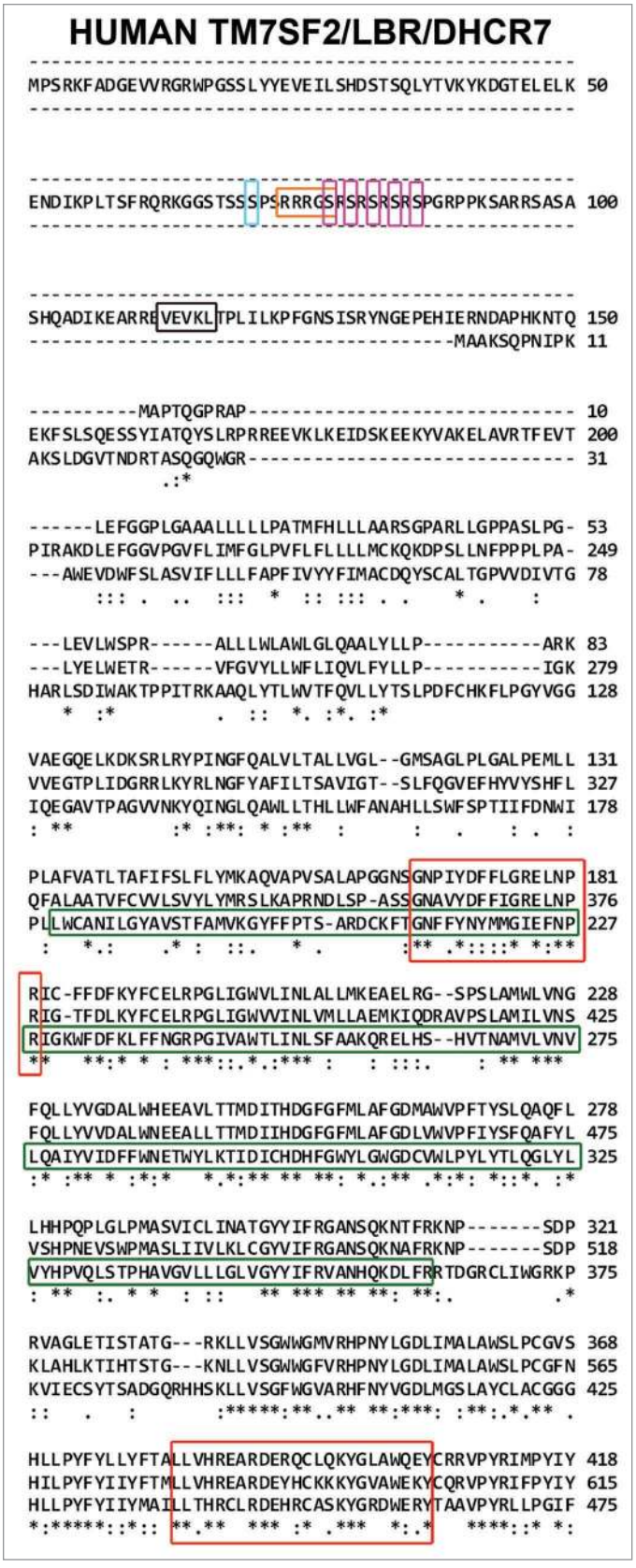

Figure 1. Clustalw 2.0,[19] Comparisons Of Human TM7SF2, LBR And DHCR7. Top peptide sequence: TM7SF2 (NP_003264). Middle sequence: LBR (NP_919424). Bottom sequence: DHCR7 (NP_001351). The sequence alignment tool was accessed through: www.ebi.ac.uk/tools/clustalw2. Vertical boxes: mitotic phosphorylation site (light blue); interphase phosphorylation sites (pink). Horizontal boxes: PKA consensus motif (orange); HP1 binding motif (black); Sterol Reductase Family Signatures 1 and 2 (red); Sterol Sensing Domain "SSD" in DHCR7 (green).

interacting with the lamina. An ortholog of chicken LBR was identified in humans[14-16] and in many other vertebrate species (see discussion on phylogenetics, below).

Examining interphase and mitotic-enriched chicken hepatocellular carcinoma DU249 cells and in vivo phosphorylation, evidence was presented that only serine residues of LBR are phosphorylated during interphase; whereas serine and threonine residues are phosphorylated during mitosis.[17] Combined with in vitro phosphorylation experiments, these authors argued that threonine in the N-terminus of LBR is the target of a cell cycle dependent $p34^{cdc2}$-type protein kinase (CDK1), perhaps as a mechanism for weakening the interaction between LBR and lamin B during mitotic NE breakdown. Simultaneous immunoprecipitation experiments on lysed avian erythrocyte NE using anti-LBR suggested the existence and binding of a third type of protein kinase acting upon LBR, distinct from PKA and CDK1 protein kinases.[18] Employing a variety of protein kinase inhibitors for in vitro studies, the authors argued that this LBR-associated protein kinase (named "p58 kinase") is also not protein kinase C or a Ca^{++}-dependent kinase. Furthermore, this p58 kinase appeared to be part of a larger complex of LBR binding proteins.

Human LBR

In two important publications,[15,16] the Worman laboratory described the human LBR protein and its encoding gene. The deduced primary protein sequence for human LBR is shown in **Figure 1** (ClustalW[19] alignment). Their study led to a number of important conclusions: (1) human LBR contains 615 aa and displays a 68% overall identity with the chicken homologue; (2) the N-terminal domain is ~208 aa and basic (pI 9.75); (3) the N-terminal domain displays consensus phosphorylation sites for PKA and CDK1; (4) the C-terminal ~407 aa contains eight predicted transmembrane segments.

GST fusion peptides from various segments of the N-terminus of human LBR, attached to glutathione-Sepharose beads, were used to examine the binding to lamins. The N-terminal 216 residues bound lamin B; but shorter fragments (i.e., residues 2–71, 2–100 and 97–216) did not. This suggests that conformational properties of the entire N-terminal region are required for lamin B binding. The study also included a gel shift (retardation) electrophoretic assay employing mixtures of the GST fusion protein (residues 2–216) and naked M13 DNA, demonstrating binding, which was destroyed following heat denaturation of the protein. A second DNA binding assay (binding of labeled DNA to nitrocellulose transfers from SDS-PAGE of the fusion proteins)

revealed that a shorter peptide region (residues 2–100) is sufficient to interact with DNA. A more recent examination of the in vitro interaction between the human LBR N-terminus and DNA and nucleosomes also employed the gel shift assay.[20] This study demonstrated that a core nucleosome with 146 bp did not interact with LBR (1–207 aa); whereas, there was clear retardation of the short (146 bp) naked DNA fragments. Longer DNA fragments (357 bp) with (or without) a core nucleosome exhibited retardation, indicating LBR binding to the "linker" region. Furthermore, the presence of histone H1 on naked or nucleosomal DNA reduced LBR (1–207 aa) interaction. It appears that, if LBR interacts with chromatin in vivo, factors not examined in this in vitro study (discussed later in this review) must be identified.

In the second Worman publication,[16] the authors present the human LBR gene sequence, which included data about the upstream promoter region. Of interest in the promoter region is the absence of TATA-like elements, the presence of putative CCAAT boxes and consensus sequences for Sp1, AP-1, AP-2 and NFκB transcription factors. The human LBR gene has 13 exons: 1–4 encode the N-terminus; 5–13 encode the C-terminus. The intron between exons 4 and 5 is large (~10 kb), suggesting "that the LBR gene may have evolved from a recombination between two primordial genes." The C-terminal sequence resemblance to several yeast proteins (including the sterol reductase *ERG24*) was noted. But the authors stated that "such a function would be unexpected for an inner nuclear membrane protein." The LBR field was in for a surprise (see later sections).

LBR Targeting Signals

The question of what structural features of LBR are responsible for its localization within the INM of interphase NE was explored in depth by studies from the Blobel[21] and the Worman[22,23] laboratories. Experiments involved transfecting mammalian COS cells with plasmids expressing a variety of constructs, including segments of chicken LBR attached to portions of ER, cytosolic proteins or nuclear proteins with nuclear localization signals (NLS). The consensus of these studies was that LBR possesses two independent non-overlapping targeting signals: one in the N-terminal ~200 aa, the other in the first transmembrane segment plus flanking residues. The N-terminal targeting signal is not the "classical" NLS of SV-40 large T antigen; but, rather, appears to resemble the bipartite NLS of nucleoplasmin (from Arg 63 to Arg 79 and from Arg 93 to Lys 108 in chicken LBR). In a persuasive experiment on the targeting property of the first transmembrane segment, residues 201–246 of chicken LBR were fused with bacterial β-galactosidase, yielding clear rim staining in the transfected COS cells. In the case of both of the identified targeting signals the nuclear presumptive binding sites are not identified. The N-terminus targeting signal seems to direct the peptide to all regions of the interphase nucleus, except the nucleolus.[22] This argues against direct binding exclusively to lamin B, which is confined to the NE. This is also not what would be expected if the targeting "receptor" is heterochromatin, since these regions are usually concentrated under the NE and surrounding the nucleolus. With respect to the binding site for the transmembrane segment, the authors[21] speculated about possible interactions with lamin B or other LBR molecules. One other feature of LBR N-terminal targeting was clearly demonstrated.[23] The N-terminal peptide must not be too large. The native N-terminus of LBR is ~23 kD. A size of ~45 kD (a chimeric protein with the native N-terminus) can still pass through the nuclear pore into the nucleoplasm; but ~70 kD is too large.

Intact chicken LBR can target to the NE in yeast (*S. cerevisiae*), independently or during co-transfection with human lamin B.[24] Earlier studies had indicated that yeast nuclei possess homologues to vertebrate lamins A and B, and LBR,[25] but this observation is now not accepted.[24] Consequently, it is not clear what features of the yeast nucleus attract the localization of heterologous LBR. Parenthetically, the same study[24] clearly demonstrated that during co-transfection, chicken LBR and human lamin B co-localized within the yeast NE, yielding in vivo support that these two proteins can interact with one another. Truncated human LBR (residues 1–238) fused to green fluorescent protein (GFP), containing only the N-terminal and first transmembrane segments LBR, has proven to be a useful tool to follow the dynamics of deposition within the NE.[26] Photobleaching microscopy of COS cells transiently transfected with truncated LBR-GFP demonstrated immobilization within the interphase NE, in contrast to a highly mobile fraction in the ER. Truncated human LBR has also been transfected into plant cells which appear not to have any obvious homologues to mammalian lamins or LBR;[27] transfection of tobacco leaf epidermal cells[28,29] yielded clear localization within the plant cell NE. The mechanism of targeting vertebrate LBR into the seemingly unrelated vertebrate, fungal or plant cell interphase NE is a subject of current research. Suffice it to say, the predominant model from the initial photobleaching study of mammalian tissue culture cells,[26] sometimes referred to as the "diffusion-retention model," envisaged that LBR can freely diffuse within the ER, the ONM, the nuclear pore complex and into the INM, where it is assumed to be immobilized by interactions with chromatin and/or the lamina.

LBR Binding Partners

Besides lamin B and DNA, a number of other putative LBR binding partners have been described. A summary of these binding partners and relevant references describing these interactions is listed in **Table 1**. During an early immunoprecipitation study[18] which identified a unique LBR (p58) kinase, a number of other proteins were observed associated with LBR in the Triton X-100 soluble complex: avian lamin B2 and A; unidentified proteins at 150, 34 and 18 kD. In a subsequent study,[30] turkey erythrocyte p34 was purified, partially sequenced and demonstrated to be homologous to mammalian p32, also known as splicing factor 2 (SF2)-associated protein. The p18 protein was characterized;[31] it appears to be an integral membrane protein unique to the avian erythrocyte NE. Evidence was presented[32] that LBR kinase phosphorylates serine residues of the RS-rich region of avian LBR. Furthermore, experiments demonstrated that phosphorylation of this RS region leads to a dissociation of p34 from the LBR complex. The LBR kinase (renamed RS kinase) was shown to modify

Table 1. LBR binding partners

Binding partner	LBR region (aa)		Reference
h lamin B*	h LBR	2–216	15
DNA (double stranded)	h LBR	2–100	15, 20
t lamin B2 t lamin A t SF2 associated Protein t RS kinase	t LBR	1–637	18, 30, 32
h HP1α	h LBR	1–208	35
h HP1γ	h LBR	97–124	40
	h LBR	113–117	43
t Histones H3 and H4 m HP1α	t LBR		45, 46
r nucleoplasmin	r LBR	54–89	47
h HA95 h LAP2β h Emerin	h LBR		48
r Protamine (phosphorylated)	h LBR		49
h MeCP2	h LBR		50
x importin β	x LBR	45–90	51

*Species designation: h, human; t, turkey; m, mouse; r, rat; x, xenopus

similar serine residues during interphase and metaphase.[32] The LBR complex isolated from avian erythrocyte NE is a fascinating association of diverse nuclear components. The function(s) of this complex are not clear. The authors[32] suggest the possibility that the LBR complex might associate nuclear "speckles" (including snRNPs) to the NE. Unfortunately, it is not clear what fraction of the total cell LBR was solubilized for the immunoprecipitation experiments. Furthermore, it is possible that some of the protein associations found in the LBR complex were generated during the extraction procedure. As a case in point: an independent study[33] presented evidence that SF2-associated protein is, in reality, a mitochondrial matrix protein and not derived from the nucleus. On the other hand, studies with human cytomegalovirus (HCMV) provide support for existence of a p32-LBR complex.[34] The large (~130 nm) HCMV capsids egress from infected cell nuclei by budding through the INM after localized disruption of the lamina. This disruption is accomplished by lamin phosphorylation following recruitment of protein kinase C to a complex of viral proteins and cellular proteins (p32 and LBR) co-localized at the NE. It should be no surprise that nuclear viruses have evolved mechanisms that can exploit existing NE protein interactions. None-the-less, these experimental uncertainties suggest the necessity of continuing to verify (or refute) the existence of a putative LBR complex in other cells and with different techniques.

In yeast two-hybrid experiments employing the N-terminal 1–208 aa of human LBR as "bait" for a HeLa cDNA library, the human heterochromatin-associated proteins HP1α and HP1γ were identified as prime candidates for LBR binding partners.[35] Furthermore, anti-LBR immunoprecipitated HP1 proteins bound to GST-LBR N-terminal domain fusion proteins. The HP1 proteins α, β and γ, originally described in Drosophila, are chromo domain proteins (mol. wt. ~30 kD) possessing an N-terminal chromo domain (CD) and a C-terminal chromo shadow domain (CSD). They are able to self-associate and, when mutated, can profoundly affect "silencing" of genetic expression, as well as chromatin modeling (reviewed in refs. 36–39). Numerous candidate binding partners for HP1 proteins have been identified, including methylated Lys 9 of histone H3 (binding to the CD), DNA replication proteins and NE proteins (binding to the CSD), emphasizing involvement in a diversity of chromatin functions. Also employing yeast two hybrid and immunoprecipitation techniques, a sequel study[40] to the initial identification of LBR-HP1 binding provided evidence that the HP1α chromo shadow domain interacts with human LBR residues 97–174. The authors applied a computational modeling procedure (HCA, hydrophobic cluster analysis[41]) to predict that the N-terminal 1–208 aa of human LBR contains two "globular domains" (residues 1–60 and 105–210) separated by a "hinge region" (residues 61–104), further suggesting that HP1 binds in the first portion of the second globular domain of LBR (residues 97–124). They also argue that the first globular domain of LBR (residues 1–60) may be critical to the interaction with lamin B, based upon an earlier analysis of LBR autoantibodies.[42] A more recent analysis[43] has proposed that there exists a common peptide motif on diverse nuclear proteins that binds to the HP1 CSD. This canonical motif is PxVxL, although alternative CSD-binding motif variants are observed (e.g., VxVxL in human LBR, residues 113–117; see **Fig. 1**). The binding of these motifs is apparently to a CSD dimer. The decreased motility of LBR in the NE compared to ER, as detected by photobleaching of GFP-LBR in live cells, may be partly due to the LBR-HP1 interaction. In a related study[44] on the mechanism of egress of polyoma virions from infected HEK293 cell nuclei, the authors demonstrated that a viral protein (Agno) dissociates HP1α from LBR (by binding to the CSD of HP1), increases the lateral motility of LBR in the NE, and facilitates viral egress without inducing nucleolysis.

In contrast, the Georgatos laboratory were unable to detect a direct in vitro interaction between the N-terminus of chicken LBR and mouse HP1 protein.[45] However, high salt/detergent extracts of turkey erythrocyte NE incubated with mouse HP1-GST yielded a complex containing HP1, histones H3/H4 and low amounts of LBR. They suggest that H3/H4 acts as a bridge, with no direct interaction between HP1 and LBR. In a more recent study,[46] the Georgatos group attempted to define the histone epigenetic markers enriched in LBR-associated chromatin. Employing a variety of nuclear extraction procedures, co-precipitation with LBR-GST, isolation of H3/H4 from SDS-PAGE gels and mass spectroscopy, they present data that H3 is heavily modified between residues 9–17 and H4 is dimethylated at Lys 20.

A number of other proteins have been implicated in binding to LBR. One study,[47] while searching for NLS binding proteins in rat liver nuclear envelopes, turned up LBR. The NLS studied is on nucleoplasmin, a nuclear phosphoprotein often associated with nucleoli and frequently described as a "chaperone." The region of rat LBR that is implicated in binding nucleoplasmin NLS spans residues 54–89, which is largely the RS region. The

binding interaction could be effectively competed by a peptide containing the SV40 large T antigen. Presently, there is no clear function of this putative LBR binding site for an NLS existing on other nuclear proteins. In another study,[48] a nuclear matrix protein (HA95) co-immunoprecipitated from detergent extracted interphase cells was complexed with LBR, LAP2β and emerin. HA95 has homology to a nuclear PKA binding protein, but does not itself bind to PKA. Immunostaining of interphase human Bjab cells indicated nuclear localization of HA95, but exclusion from nucleoli. The authors argue that HA95 anchors the nuclear envelope to chromatin in interphase nuclei. More recently, evidence has been presented that phosphorylated protamine can bind to LBR during spermiogenesis in the rodent testis.[49] The authors view this observation as demonstrating that LBR plays a role in the orchestrated transition from histones to protamines, which may also involve the RS protein kinase[32] and p32.[30] A provocative recent study[50] presents evidence that a methylated DNA binding protein (MeCP2) binds directly to LBR, thus bringing certain heterochromatic regions into proximity to the NE. Most interphase MeCP2 is distributed throughout the nucleus, with a small fraction co-localized with LBR. Both MeCP2 and LBR are present in a pellet fraction, prepared by sequential micrococcal nuclease of isolated HeLa nuclei, followed by salt/detergent extraction. Constructs of MeCP2, interacted in vitro with immobilized LBR and in vivo using a "bimolecular fluorescent complementation" assay, demonstrated that the "linker" region of MeCP2 (residues 162–202 aa) is necessary for the binding interaction. In another important paper,[51] evidence was presented supporting the binding of importin β to the N-terminus of Xenopus LBR, resulting in the targeting of NE membrane precursors to chromatin (see below). Employing various GFP-LBR constructs to bind to importin β-coated beads, the authors argued that the Xenopus LBR binding region spanned residues 45–90, corresponding to residues 41–81 in human LBR (note our correction of their residue assignments), which includes the last portion of the first "globular" domain of LBR and part of the "hinge" region. The authors also presented evidence that Ran GTP binding to the importin β-LBR complex may lead to dissociation of the complex.

One other post-translational modification of LBR should be described in this review. *O*-linked β-N-acetylglucosamine (*O*-GlcNAc) of Ser 96 in rat liver LBR has been reported.[52] Since this a dynamic modification, it implies the binding of LBR to *O*-GlcNAc tranferase and, at other times, to *O*-GlcNAcase. The significance of this modification is unclear. It is within the RS region and could be a site of phosphorylation (although it is not a site expected for the RS protein kinase or CDK1 kinase). Perhaps more difficult to explain, this serine residue is not conserved in homologous chicken or human LBR.[47] It would be important to examine homologous LBR molecules to see whether this post-translational modification can be identified at other serine residues and under various cellular conditions.

Surveying the numerous studies of LBR binding partners illustrates a few recurring themes. A number of heterochromatin associated proteins (HP1 and MeCP2) have been shown to interact directly with LBR. One possible transcription-related protein (SF2-associated protein) is also found in complex with LBR. The binding studies are complicated by the insolubility of LBR, requiring assays to frequently be based upon sepharose-immobilized portions of LBR, or the use of NE extracts that probably only partially recover the total nuclear LBR. A recurrent theme is the phosphorylation of the RS region of LBR, which appears to modulate interactions with the various binding partners. For obvious reasons, binding experiments have been confined to the nucleoplasmic N-terminus of LBR. Technical difficulties currently prevent exploration of binding to the NE membrane-embedded C-terminus of LBR.

The Cell Cycle and LBR

In eucaryotic cells with "open" mitosis, the nuclear envelope begins to break down in prophase and starts to reform in late anaphase (see reviews on the NE and chromatin changes at mitosis[53-55]). Breakdown of the NE involves a series of macromolecular changes, including dispersal of the nuclear pore complexes, depolymerization of the lamina, retreat of the NE membranes into the ER, and beginning condensation of the mitotic chromosomes. Underlying these changes are numerous biochemical events; one of the best studied being the widespread phosphorylation of pore proteins, lamins, inner-nuclear membrane proteins and chromatin. A number of kinases appear involved in these processes, primarily the cyclin-dependent kinase (CDK1). The role of phosphorylation in dismantling the NE and associated heterochromatin is not entirely understood; as exemplified by the LBR-lamin B-chromatin interaction, the binding may be weakened favoring dissociation.[17,56,57] However, in the studied case of chicken LBR, the CDK1 site(s) of mitotic phosphorylation are in dispute: one group[17] identified Thr 188 as the site and suggested that this might destabilize the LBR-lamin B interaction; the other group[57] identified Ser 71 and argued against a weakening of LBR-lamin B binding. More recent studies,[58,59] involving the binding of Xenopus sperm chromatin to LBR-GST beads, present evidence that during S phase the RS protein kinase stimulates LBR-chromatin binding by phosphorylation of one or more serine residues in the RS region (see **Fig. 1**). Furthermore, they identify Ser 71 as the phosphorylation site by mitotic CDK1 kinase, which results in a weakening of the LBR-chromatin interaction.

During mitosis, LBR is retained within the retreating ER and kept out of the way of the mitotic apparatus. Early studies on mitotic HeLa cells,[60] chicken hepatoma cells[61] and human lymphoblasts[62] suggested that the interphase cell NE/ER breaks down into vesicles. Fractionation techniques provided evidence that LBR-containing vesicles remained associated with lamin B (but not lamin A)[61] and with LAP2β.[62] The nuclear pore protein (gp210) was found in a different population of vesicles.[60] Data were also obtained supporting that sea urchin eggs contain LBR in vesicles that are devoid of lamin B.[63] With respect to mitosis in higher eucaryotic tissue culture cells, the conception of the mitotic ER has drastically changed. The ER membrane system is now regarded as an intact network, with little or no vesicle formation.[26] Inner NE proteins (LBR, LAP1 and 2, gp210) are viewed being able to rapidly disperse throughout the entire mitotic ER

network.[26,64] Vesicle formation in the extracts of mitotic cells is now regarded as a fragmentation artifact of the cell lysis procedure, see also discussions;[65,66] but cytoplasmic vesicles containing NE components in (sea urchin) eggs remains a possibility. Furthermore, the current view is that as the NE membrane sheets withdraw from condensing chromosomes, the ER sheets transform into tubular structures[67] due to the addition of integral membrane proteins (reticulon and DP1/Yop1) that promote ER tubules.[68]

During post-mitotic nuclear reformation, there appears to be an orderly sequential binding of NE protein components, attachment of ER membrane sheets, closure of the membranes, reconstruction of the nuclear pore complexes, and decondensation of the mitotic chromosomes. The inactivation of mitotic kinases, coupled with the protein phosphatase activity, result in dephosphorylation of lamins, NE membrane and nuclear pore components, and mitotic chromosomal proteins.[55,69] NE reformation begins in late anaphase and is completed in telophase. LBR appears to play a special role in the NE reformation process. Early studies on the deposition of NE components upon post-mitotic chromosomes favored the conclusion that LBR containing vesicles deposit on chromatin before lamin B containing vesicles in HeLa[60] and sea urchin[63] cell-free systems, in disagreement with the observation that LBR and lamin B associate within the same vesicle derived from chicken hepatoma cells.[61] In an important study of post-mitotic HeLa cells[70] involving fluorescent NE proteins, data supported the following sequence of binding to chromatin: during late anaphase, LBR, emerin and LAP2 accumulate; during telophase, some nuclear pore components accumulate; from late telophase to the beginning of G_1, lamins A and B bind, as well as gp210; the NE is sealed and nuclear pores become functional. Microscopic images[53,71] of late anaphase binding of LBR, emerin, LAP2α and β support that these proteins initially localize at different chromosome regions. LBR and LAP2β are first observed at the lateral "peripheral" margins of the separating chromosomes, as defined by the spindle "pole-to-pole" axis. LAP2α and emerin concentrate within the central "core" region. By the end of telophase, these discrete localizations disappear; the NE proteins display a more uniform distribution. Correlative light and electron microscopy has added greatly to our description of the post-mitotic time course of events.[72] Image data strongly supports the initial differential distributions of NE components around the reforming NE. BAF, a chromatin binding protein that also binds to a common peptide motif ("LEM domain") on LAP2β, emerin and MAN1, concentrates on the central core region attaching to spindle microtubules and apparently excluding the initial formation of NE membranes, which occurs in the peripheral chromosome region, where membrane-associated LBR is being deposited.

Employing a Xenopus egg extract to examine NE formation around Xenopus sperm chromatin, evidence was presented that protein phosphatase 1 (PP1) is responsible for LBR dephosphorylation at Ser 71,[73] and targeting of membrane vesicles to the chromatin. Targeting was blocked by prior treatment with anti-PP1 or by addition of excess amounts of an LBR RS-region peptide. These results are nicely consistent with the earlier published view that LBR is responsible for the "docking" of turkey erythrocyte NE membrane vesicles to chromatin.[74] Phosphorylation of Ser 71 by CDK1 is a mitotic event, since it is not identified on interphase LBR; other serine residues (avian LBR: 76, 78, 80, 82, 84) are targets of the RS protein kinase and do not show obvious cell cycle changes[57] (see earlier mention of corroborating experiments with Xenopus egg extracts and sperm chromatin[58,59]). PP1 appears to play a role in lamin B polymerization within HeLa G_1 cells.[75] The level of phosphorylation of HP1 also reveals some cell cycle variation, which correlates with their nuclear localization: HP1α and γ increase their level of phosphorylation during mitosis; HP1β does not.[76] Although there is evidence that phosphorylation of HP1γ at Ser 83 impairs its silencing activity and favors its localization in euchromatin,[77] there is no information about influences on the binding to LBR. It is clear that much still needs to be unraveled concerning the relationship between cell cycle-dependent post-translational protein modifications and the integrity of the LBR-lamin B-HP1-heterochromatin complex. As mentioned earlier, evidence has been presented that importin β binds to the N-terminus of LBR, mediates the interaction between membrane bound LBR and chromatin, and dissociates from LBR following Ran-GTP hydrolysis.[51] There is currently no information about whether the stability of the importin β-LBR is influenced by phosphorylation within the LBR RS region.

LBR Influences on Nuclear Shape and Heterochromatin Distribution in Myeloid Cells

Higher eukaryotic cells usually possess nuclei that are round or oval in shape,[9] and position much of the silenced heterochromatin at the nuclear periphery.[7,78] There are exceptions to this generalization: (1) Blood granulocytes in vertebrates are generally lobulated or ring-shaped.[79] (2) Rod photoreceptors of nocturnal (but not diurnal) mammals possess the bulk of heterochromatin in the center of the nucleus, with the euchromatin at the periphery.[80] The unusual nuclear shape of blood granulocytes is now well studied. Biochemical, cell biological, and human and mouse genetic evidence[79,81-87] supports the contention that sufficient levels of LBR are required during in vivo and in vitro granulopoiesis (differentiation of granulocytes) in order to achieve the unusual nuclear shape. Other factors also appear to play a role in determination of granulocyte nuclear shape; i.e., a paucity of lamin A/C and B1,[82] and the presence of intact microtubules.[88] In the absence of sufficient LBR, the differentiated granulocyte nuclei are ovoid, appear to have a reduced NE surface area, and the heterochromatin redistributes towards the center of the nucleus.[84-86] In vitro granulopoiesis of the human acute myeloid leukemia cells (HL-60) with retinoic acid results in a marked increase of LBR content and nuclear lobulation with an exaggerated growth of the NE into sheets (nuclear envelope-limited chromatin sheets, or "ELCS"[81,89]). It should also be mentioned that overexpression of the C-terminal region of LBR following transfection of HeLa cells results in perinuclear aggregates, stacks of overproduced membranes pinched off the NE,[51] which are devoid of lamin B. Even though these stacks do not resemble ELCS, which contain both lamin B and

chromatin, the evidence is consistent with the notion that LBR is capable of stimulating NE membrane growth. The problem of induction of membrane growth is discussed in the last section of this review.

The connection between LBR, nuclear shape and heterochromatin distribution acquired credence from studies of human and mouse genetic mutations affecting granulocyte nuclei. Human Pelger-Huët anomaly (PHA) is a hematological condition whose history, genetics and clinical characteristics has been recently reviewed.[79,90,91] Heterozygous PHA is a rare condition (0.01–0.1% of the population), where (on blood smears) the majority of neutrophil granulocytes exhibit a bilobed appearance, rather than the normal 3–4 lobes. The clinical significance is distinguishing heterozygous PHA from potentially more serious, but similar appearing "pseudo-Pelger" morphologies, such as seen in various infections, neoplasias and following certain medications.[90-92] Homozygous PHA is considerably more devastating; most such individuals do not come to "term" (birth). Blood smears and thin section electron microscopy reveal that the neutrophil nucleus is ovoid with heterochromatin redistribution.[79] Clinically, homozygous PHA presents a varied picture, sometimes exhibiting skeletal defects, developmental problems and mental retardation.[93] The condition is less severe in rabbits and mice. The analogous genetic condition to human PHA in mice is called "ichthyosis (*ic*)" and has been clearly shown to result from a deficiency of LBR.[86] The heterozygous blood phenotype is less severe in mouse *ic* than in human PHA: the heterozygous mouse neutrophil nucleus looks normal (i.e., ring-shaped); whereas, the heterozygous human neutrophil nucleus is bilobed. Homozygous *ic* mice exhibit ovoid neutrophil nuclei on blood smears, with marked redistribution of heterochromatin toward the center. Other cell types in homozygous *ic* mice exhibit similar heterochromatin redistribution (e.g., splenic lymphocytes). As with human PHA, most of the homozygous *ic* fetuses never come to term. But unique to mouse ichthyosis, homozygous animals exhibit a distinctive loss of hair and hyperkeratosis, not seen in PHA. Clearly, the blood granulocyte nuclear changes make some sense in terms of the view of LBR "stitching" together NE components and heterochromatin; but the developmental problems and high fetal mortality imply that other LBR functions might be responsible.

A phenocopy of mouse ichthyosis was generated using a "gene-trap insertion" into the mouse LBR gene.[94] The mutation yielded a hybrid protein consisting of the N-terminal 366 aa (containing the first four TM segments and missing the last four TM segments of the C-terminus) fused to β-galactosidase. This LBR-β-gal fusion protein was mislocalized into the nucleoplasm and ER of gene-trapped fibroblasts. In addition, the gene-trapped fibroblast nuclei were misshapen and LAP2β and HP1 were mislocalized. The HP1α positive nuclear regions (focal chromocenters in mouse cells) were larger and fewer in number, exactly as reported in LBR deficient mouse granulocytic EPRO cells.[85]

The Sterol Reductase Properties of LBR

The 1994 description of human LBR[16] noted the similarity of the C-terminus to sterol reductase, but questioned whether such a function was expected within the inner membrane of the NE. In a subsequent study,[95] the Worman group identified two paralogs in the human genome for the C-terminus of human LBR. Both of these genes code for proteins that are devoid of the basic N-terminal ~200 aa of intact LBR. The predicted protein sequences are remarkably similar to the C-terminus of LBR: TM7SF2 (also called DHCR14 or SR-1), has 58% identical and 75% conserved aa residues with LBR and exhibits C-14 sterol reductase activity when overexpressed in COS-7 cells;[96] DHCR7 (SR-2) has 37% identical and 62% conserved aa with LBR. So far, the only function ascribed to TM7SF2 is the C-14 sterol reductase activity. However, a number of cited articles have demonstrated that DHCR7 is involved in the conversion of 7-dehydrocholesterol to cholesterol, and when mutated, is the genetic basis of the Smith-Lemli-Opitz syndrome. **Figure 1** presents an alignment of human TM7SF2 with human LBR and DHCR7. A phylogenetic tree constructed in an earlier study,[95] illustrated that LBR and TM7SF2 have a closer evolutionary relationship than LBR and DHCR7, and that human DHCR7 is more closely related to a sterol reductase from Arabidopsis than to human LBR and TM7SF2. Employing sterol synthesis mutants in *S. cerevisiae*, it was shown that human LBR complements C14 sterol reductase (*ERG24*), but not C24(28) sterol reductase (*ERG4*).[97] The authors speculate that LBR may be involved in cholesterol biosynthesis in the NE, or might be a receptor for cell cycle signaling sterol molecules. Another investigation[98] demonstrated complementation of mutations in the *erg-3* gene in *N. crassa* (a C-14 sterol reductase) by the C-terminal region of human LBR or by TM7SF2. They also speculated on a possible role of sterols in cell cycle changes of the NE.

Confirmation of a clinical significance to the C-terminal sterol reductase portion of human LBR appeared in 2003.[99] While studying a rare autosomal recessive in utero lethal syndrome (HEM/Greenberg skeletal dysplasia), the authors demonstrated that skin fibroblasts from an 18-week-old fetus with this condition accumulated a sterol precursor intermediate in cholesterol biosynthesis (cholesta-8,14-dien-3β-ol), not seen in fibroblasts from healthy fetuses of the same age. They ruled out TM7SF2 as the defective C-14 sterol reductase and demonstrated that the fetal tissues possessed a homozygous stop codon in the LBR gene, with a predicted truncation of the C-terminal 82 aa. Additionally, the mother of the HEM fetus exhibited the classical PHA granulocyte nuclear bilobed appearance, indicative of heterozygosity. Unfortunately, no data was available concerning the father. Implication of LBR, rather than TM7SF2, in cholesterol biosynthesis is even more remarkable, since all of the other enzymes in the post-squalene-to-cholesterol pathway reside in the ER (as does TM7SF2[96]). Summaries of the variety of known human malformations resulting from genetic defects in cholesterol biosynthesis have been published.[100,101]

It appears that heterozygous mutations of the human LBR gene exhibit a largely benign dominant trait (hypolobulation of granulocyte nuclei); but in the homozygous state, the consequences can be far more devastating. A review[93] of the phenotypes of homozygous PHA compared to HEM/Greenberg dysplasia reveals that there is a wide clinical spectrum: most reported

Table 2. LBR mutations

Nucleotide change	Location	Amino acid substitution	Mutation class	Reference
		Pelger-Huet Anomaly		
IVS2-2A→G	intron 2		splice acceptor	84
IVS12-5-10del	intron12		splice acceptor	84
1308G→A	exon 10	Trp436X	nonsense	84
IVS11 + 1G→A	intron 11		splice donor	84
1173delC	exon 9	Gly392fsX393 (Gly392Asp, Leu393X)	frame shift	84
1129C→T	exon 9	Arg377X	nonsense	84
IVS13-2A→G	intron 13		splice acceptor	84
500G→C 501-504delCCTT	exon 5	Ser167fsX176 (Ser167Thr, Lys176X)	frame shift	84
Cd119CCG→CTG	exon 3	Pro119Leu	missense	103
IVS11-9 A→G	intron 11		splice donor	103
Cd569C→G	exon 14	Pro569Arg	missense	103
		HEM/Greenberg Skeletal Dysplasia		
1599-1605 TCTTCTA→CTAGAAG	exon 13		stop codon	99
1639 A→G	exon 13	Asn547Asp	missense	102

PHA homozygotes display few congenital abnormalities; HEM/Greenberg dysplasia exhibits severe skeletal defects and in utero lethality (see also, recent descriptions[102]). However, it should be mentioned that in only one case of homozygous PHA is the genetic background clear. The other "homozygous" PHA individuals were diagnosed by their neutrophil nuclear morphology. Analysis of the underlying mechanisms for the diverse phenotypes has not been straight forward. All of the analyzed mutations producing HEM/Greenberg dysplasia are in the C-terminal sterol reductase region of LBR;[93,99,102] but many of the mutations of the proven PHA (including the surviving homozygous male adult) are also in the sterol reductase region.[84,103] **Table 2** presents a list of published PHA and HEM/Greenberg dysplasia mutation sites.

Two recent studies[104,105] have generated a TM7SF2 "knockout" in mice in order to compare its phenotype with *ic* mice. Both studies have concluded that TM7SF2 (-/-) mice develop and appear normal, exhibit no obvious pathologies and have normal cholesterol biosynthesis in the liver. It appears that the LBR C-14 sterol reductase activity is functionally redundant and adequate for the animal's needs. By contrast, in *ic* (-/-) mice, which exhibit high fetal mortality, developmental pathologies and shortened lifespan, normal levels of TM7SF2 can not compensate for the LBR deficiency.[86,104] By intercrossing heterozygous *ic* (+/-) with heterozygous TM7SF2 (+/-) mice, it was shown[104] that a lower level of LBR in *ic* (+/-), TM7SF2 (-/-) mice was not enough to keep the animals normal; they developed neurological symptoms and died at ~14 days due to damaged myelin sheaths of the spinal cord. This phenotype, however, does not resemble mouse ichthyosis or human HEM/Greenberg dysplasia. It was concluded that, although there is sterol reductase redundancy between LBR and TM7SF2, ichthyosis and HEM/Greenberg dysplasia are "laminopathies" resulting from other interactions and functions.

LBR Polypeptide Conformations and Conserved Domains

The distinction between the (nucleoplasmic) basic N-terminal ~208 aa and the C-14 sterol reductase-like C-terminal ~407 aa is fundamental. A major advance in the structural analysis of LBR has been the suggestion[106] and subsequent confirmation by NMR[107] that the N-terminal globular region (residues 1–60) is a member of the Tudor domain "Royal Family."[108] Besides the NMR solution structural data on the human LBR Tudor domain, there have been NMR and crystallographic studies on a number of other Tudor domain-containing proteins complexed to binding ligands: (1) *D. melanogaster* Tudor-SN[109] (related to human SMN[110]), which specifically binds a peptide containing symmetrical dimethylated arginines (sDMA); (2) mammalian DNA repair factor 53BP1, which possesses two tandem Tudor domains that bind to a single dimethylated lysine of histone (H4K20me2), but do not bind H4K20me3;[111] (3) human histone demethylase JMJD2A, a tandem "interdigitated" Tudor domain protein that can bind H4K20me3 or H3K4me3.[112,113] **Figure 2** presents images of the LBR Tudor domain (Protein Data Bank PDB: 2dig; see also **Fig. 5A**) and images of Tudor domains from 53BP1 (PDB: 2ig0), JMJD2A (PDB: 2qqs) and Tudor-SN (PDB: 2wac). By comparing these images (in 3-D) and analyzing specific residues and their positions within the "aromatic cage," we suggest that the human LBR Tudor domain most resembles 53BP1, and probably binds H4K20me2. It is tempting to speculate that such an interaction might promote LBR-heterochromatin interactions by binding to nucleosomes with this specific histone modification.

The human LBR peptide region identified as "hinge,"[40] spanning residues 61–104, encompasses the RS-rich region and is the major location of interphase and mitotic phosphorylation.[57-59,73] RS domain-containing proteins are very prevalent in metazoan nuclei,

where they are involved in numerous functions, including RNA polymerase II transcription, pre-mRNA splicing, nuclear export and translation.[114-117] Given the likely interaction of LBR with repressed (transcriptionally-inactive) chromatin, an interaction with nuclear RNA would seem to be unexpected. However, a recent study[118] argues that nuclear RNA association with the RS domain of chicken LBR is important for disaggregation of LBR oligomeric complexes present in the inner nuclear membrane. A similar disaggregation can be accomplished by binding to DNA or by phosphorylation with the RS protein kinase (SRPK1). Thus, the RS domain may be involved in LBR structure and function, being in part regulated by RNA binding or phosphorylation. An additional point in this article[118] derives from bioinformatics predictions of chicken LBR peptide "order/disorder" in the N-terminal ~200 aa: the region from ~61–104 is highly disordered, whereas the Tudor domain region is highly ordered. The authors of the present review have examined peptide order in the "hinge" region of human LBR, employing ExPASy ELM GlobPlot (http://elm.eu.org) and concur that residues 60–95 are predicted to be highly disordered (i.e., not globular). Similarly, the human LBR 125–143 region is highly disordered; but a globular domain is predicted for residues 144–237. Other than the predicted motif for binding the HP1 CSD[43] (residues 113–117), very little can be said about the conformation of the ~100–200 region.

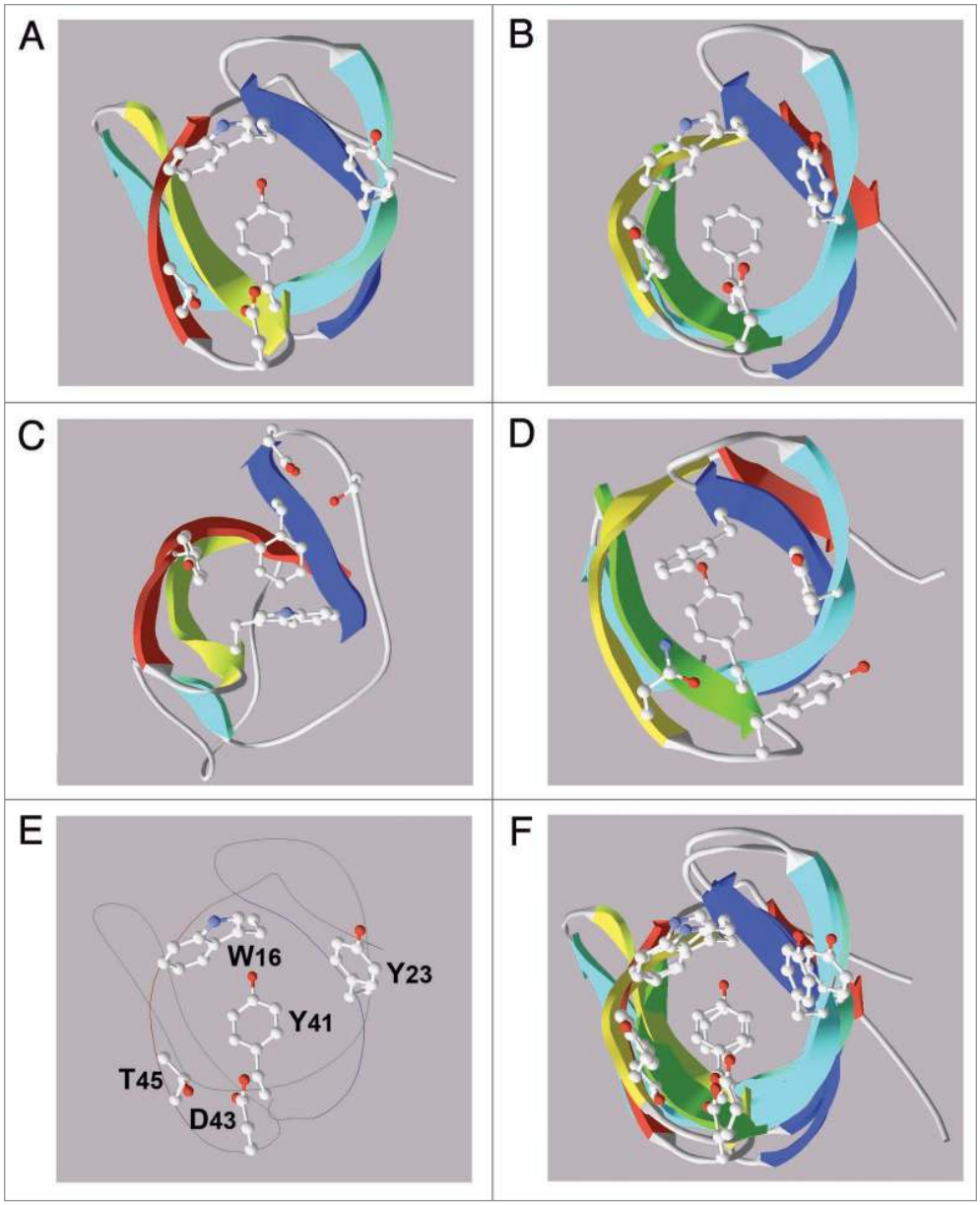

Figure 2. Structural comparisons of tudor domains in various proteins. All of the structures were obtained from the Protein Data Bank "PDB" (www.rcsb.org/pdb/) and were oriented and modified using "Deep View" (http://spdbv.vital-it.ch/index.html). (A) LBR Tudor domain (PDB: 2dig); (B) 53BP1 (PDB: 2ig0); (C) JMJD2A (PDB: 2qqs); (D) Tudor-SN (PDB: 2wac). The bottom left image (E) shows only the putative binding sidechains in the aromatic cage of 2dig. The bottom right image (F) shows a superposition of 2dig and 2ig0. Arrow heads are the C-termini of β-sheets. Each element of secondary structure is assigned a color, progressing dark blue, light blue, green, yellow, red as the peptide chain progresses from N-to-C-terminus.

The C-terminal region (~407 aa) of human LBR is characterized by predicted transmembrane (TM) segments and observed C-14 sterol reductase activity. Bioinformatic searches and tools provide both inconsistent predictions and frustrating suggestions of conserved motifs and domains. Employing many of the TM prediction tools available on ExPASy (**Suppl. 1**), we obtained clear disagreement on the total number of TM segments: of the seven tools tested on human LBR, four predicted 8 TM; three predicted 9 TM. There was some agreement, however; seven TM segments were predicted in nearly identical positions by all the methods. **Figure 3A** presents the graphical output of the highly regarded[119] TMHMM program available through ExPASy or CBS (www.cbs.dtu.dk/services/). This program predicts 8 TM segments and specifies the direction of the TM segment (i.e., in the case of LBR, "inside" refers to the nucleoplasm; "outside" refers to the lumen between the INM and ONM). **Figure 3B** schematically represents the conformational consequences of LBR possessing 8 or 9 TM segments: 8 segments would position the N-terminal ~208 aa and the C-terminal post-TM ~38

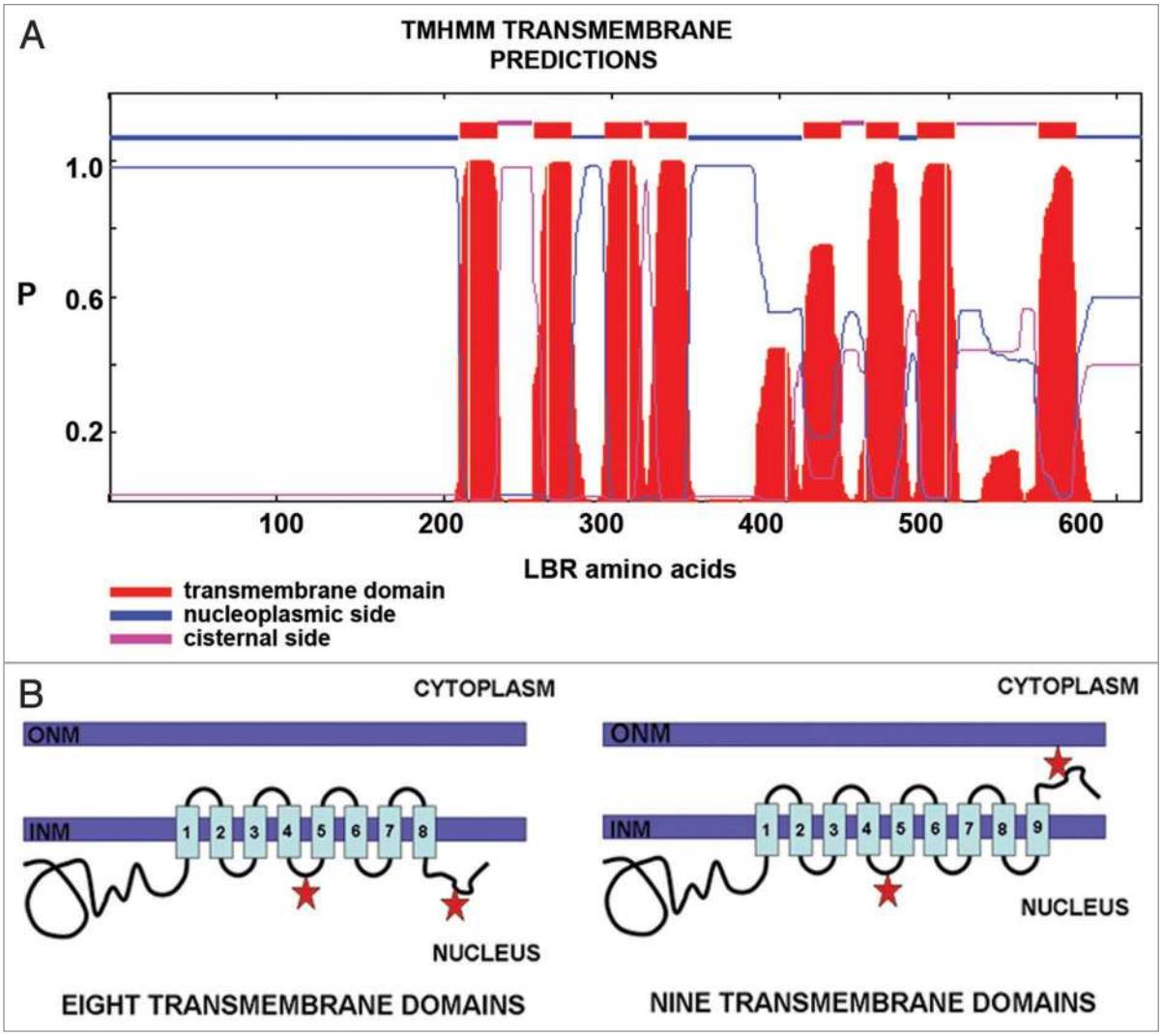

Figure 3. Predicted transmembrane (TM) structure of human LBR. (A) TMHMM program from CBS (www.cbs.dtu.dk/services/) applied to human LBR (NP_919424). Axes: P, probability of TM domain; LBR amino acids, 1–615. The upper line displays the positions of the predicted TM domains (red), the nucleoplasmic side of the INM (blue) and the perinuclear cisternal side (pink). Note the very strong predictions for the first 4 TM domains. (B) Scheme demonstrating the consequences of 8 versus 9 TM domains to the disposition of the C-terminal "tail." The red asterisks denote the sterol reductase signatures 1 and 2 (shown in **Fig. 1**). It should be emphasized that this cartoon represents the sequence of TM domain laid out in a line, an unlikely positioning. More likely would be different orientations and possible interactions between the various TM domains (shown in **Figs. 5** and **8**).

aa within the nucleoplasm; 9 segments would flip the C-terminal "tail" into the lumen between the ONM and INM. The significance of this conformational difference is unfortunately obscure, since no function has been ascribed to the C-terminal tail.

Employing ExPASy (Pattern and Profile Search, ScanProsite) with human LBR (Swiss-Prot Q14739) returned two motifs within the C-terminus described as "Sterol Reductase Family Signatures 1 and 2" with homologies to many sterol reductases, including yeast ERG 4 and 24 and human TM7SF2 and DHCR7: signature number 1 spans from residues 362–377; signature number 2 spans from residues 579–602 in human LBR (see **Fig. 1**). Superimposing these residue positions upon the TMHMM prediction (**Fig. 3B**), both signatures would be within the nucleoplasm; but 9 TM segments would flip signature 2 into the perinuclear cisternae.

An additional feature associated with cholesterol homeostasis, named "sterol-sensing domain (SSD)" has been identified in a number of proteins, including DHCR7 and HMG Co-A reductase (HMGCR).[120-123] The SSD is highly conserved with representatives found ranging from humans to nematodes. The consensus topology is described as consisting of ~180 amino acids organized into a cluster of five consecutive TM domains. Examining a ClustalW pairwise alignment of human HMGCR with human LBR indicated that the putative HMGCR SSD region (residues 57–230) did not align with the C-terminus of human LBR (data not shown). However, a potential SSD of human DHCR7,[120] spans residues 181–362, encompassing Sterol Reductase Family Signature 1. Comparing the aligned human DHCR7 and human LBR (**Fig. 1**), the LBR SSD could extend from residues 330–512, a region including ~4 TM segments. In addition, a short sequence

feature associated with the SSD is the tetrapeptide YIYF, observed in human HMGCR, SCAP and other proteins, with a variant (YYIF) seen in human DHCR7.[122] The sequence YYIF is also seen in human TM7SF2, with YVIF observed at a corresponding position in human LBR. (Parenthetically, it should be noted that the final 3 residues of human LBR and TM7SF2 are "YIY"). The SSD appears to respond to varying levels of cellular sterol by modifying protein conformation and function, probably acting as a regulatory domain. At this time, there is no convincing indication that LBR actually possesses an SSD, nor what regulatory role it might play in LBR function.

Both Pfam and the NCBI Conserved Domain Database (CDD) identify the entire C terminal domains of LBR, DHCR7 and TM7SF2 as members of the ERG4_ERG24 family of ergosterol biosynthesis domain (e.g., residues 205–615 of human LBR match the domain with expectation values of 5e-221 and 1e-147 using hmmpfam to Pfam_fs and blastp to the CDD, respectively). This domain is a member of the ICMT (isoprenylcysteine methyltranferase) CDD superfamily, and the last ~100 amino acids of LBR have a significant match to the ICMT conserved domain (residues 521–615 of human LBR have an expectation value of 9e-8). Human ICMT is an integral membrane protein present within the ER. It has 284 aa and contains 8 predicted TM segments with the N and C termini facing the cytosol.[124] Only one gene for the ICMT class of methyltransferases is present within the sequenced mammalian genomes. Deletion of ICMT in mice results in embryonic lethality.[125] ICMT operates on a class of proteins that terminate with a CaaX motif (C denotes cysteine; a, any aliphatic aa; X, any aa). Following prenylation of the cysteine, a protease (Rce1) removes the aaX and ICMT methyl esterifies the isoprenylcysteine carboxyl moiety. Of the target CaaX group of proteins, the most prominent is the RAS superfamily of GTPases, whose numerous mutations and alterations of expression are associated with a variety of cancers. Consequently, the enzymes involved with isoprenylation, proteolysis and methyl esterification are major targets for anticancer drugs.[126] Lamins are another group of CaaX proteins that are modified like the RAS proteins, promoting their interaction with the membranes of the NE, although mature lamin A does not maintain this functionality. Recent studies employing embryonic mouse fibroblasts made null for Rce1 or ICMT suggest that carboxymethylation of lamin B1 may not be essential for a normal lamina.[127] However, it is conceivable that the ICMT "property" of the LBR C-terminus (or, indeed, a "property" in TM7SF2 and DHCR7) may be redundant for the lamins, compensating for loss of the bonafide ICMT. As yet, the significance of the homology between ICMT and the C-terminus of LBR remains a mystery, since no methyltransferase activity has ever been demonstrated with LBR.

The Phylogenetics of LBR

Analysis of the human LBR gene suggests that a recombination of two different genes (one related to sterol reductases) might have been involved in the evolution of the LBR gene.[16] In a recent study of the evolution of the nuclear envelope and nuclear pores employing bioinformatic tools,[106] the authors support this contention (also mentioning the Tudor domain as part of the N-terminus) and suggest that LBR is unique to vertebrates. This latter conclusion reflects the more limited database of completely sequenced eukaryotic genomes available at that time (2004). We searched NCBI databases and currently available genomes for copies of DHCR7, TM7SF2 and LBR. We found that DHCR7 is present in all three major multicellular groups (animals, fungi and plants), TM7SF2 is present only in some vertebrates, and LBR is present in at least some non-vertebrate deuterostomes (**Fig. 4**). TM7SF2 appears to have originated through a gene duplication early in vertebrate evolution and to have been subsequently lost in some lineages, as we were unable to locate a copy of the gene in the complete or nearly complete genomes of the chicken or the platypus (although we were able to locate complete copies of LBR and DHCR7). The relationship of LBR to DHCR7 shown in **Figure 4** suggests that at least the C-terminal region of LBR is more ancient than deuterostomes. **Supplement 2** presents the evidence for designating as "true" the LBR orthologs found in two non-vertebrate deuterostomes: the sea squirt, an ascidian chordate, and the sea urchin, an echinoderm. LBR genes in both species are similar to human LBR across their entire length and both contain N-terminal Tudor domains, two conditions lacking in the DHCR7 orthologs identified in both genomes. We also identified potential LBR homologs in the partial genome sequences currently available for the cephalochordate *Branchiostoma floridae* and the hemichordate *Saccoglossus kowalevskii*; however, we were only able to annotate the C-terminal ERG4_ERG24/ICMT region (and were also only able to locate a fragment of DHCR7 in *S. kowalevskii*). As there is no collaborating mRNA evidence, we cannot conclude whether the cephalochordate and the hemichordate genes lack the Tudor domain region, or if the sequence assembly is incomplete, or if a large intron thwarted our attempt to annotate the full-length coding sequences. Identification of potential LBR genes has not been extended to mollusks, annelids, cnidarians (coral, anemones and jellyfish), sponges or comb jellyfish. Hopefully, with the completion of more genomes in different taxa, resolution of the evolutionary emergence of a recognizable LBR will become even clearer. It is also worth pointing out that the residues of the LBR Tudor "aromatic cage," involved in the presumptive binding of H4K20me2, appear to be very highly conserved (**Table 3**). This, combined with conservation of the C-terminal structure, argues that the function of LBR has remained conserved, at least since the appearance of the deuterostomes.

The issue of an arthropod LBR is a bit more problematic. The best studied putative arthropod LBR is from Drosophila, "dLBR."[128] Similarities with vertebrate LBR are that this protein is basic (pI = 9.83) with a C-terminus (residues 307–741) containing 8 putative TM regions. Furthermore, specific antibodies demonstrate that dLBR is localized to the INM, and that the N-terminus binds to Drosophila lamin Dm0 and to Xenopus sperm chromatin. However, there is no discernible phenotype when silenced by RNAi.[128] We estimate that Drosophila LBR shares "marginal" 24% identity with residues 110–615 of human LBR and has a match to the ERG4_ERG24 domain at residues 313–653 (**Suppl. 2**). However the match to the

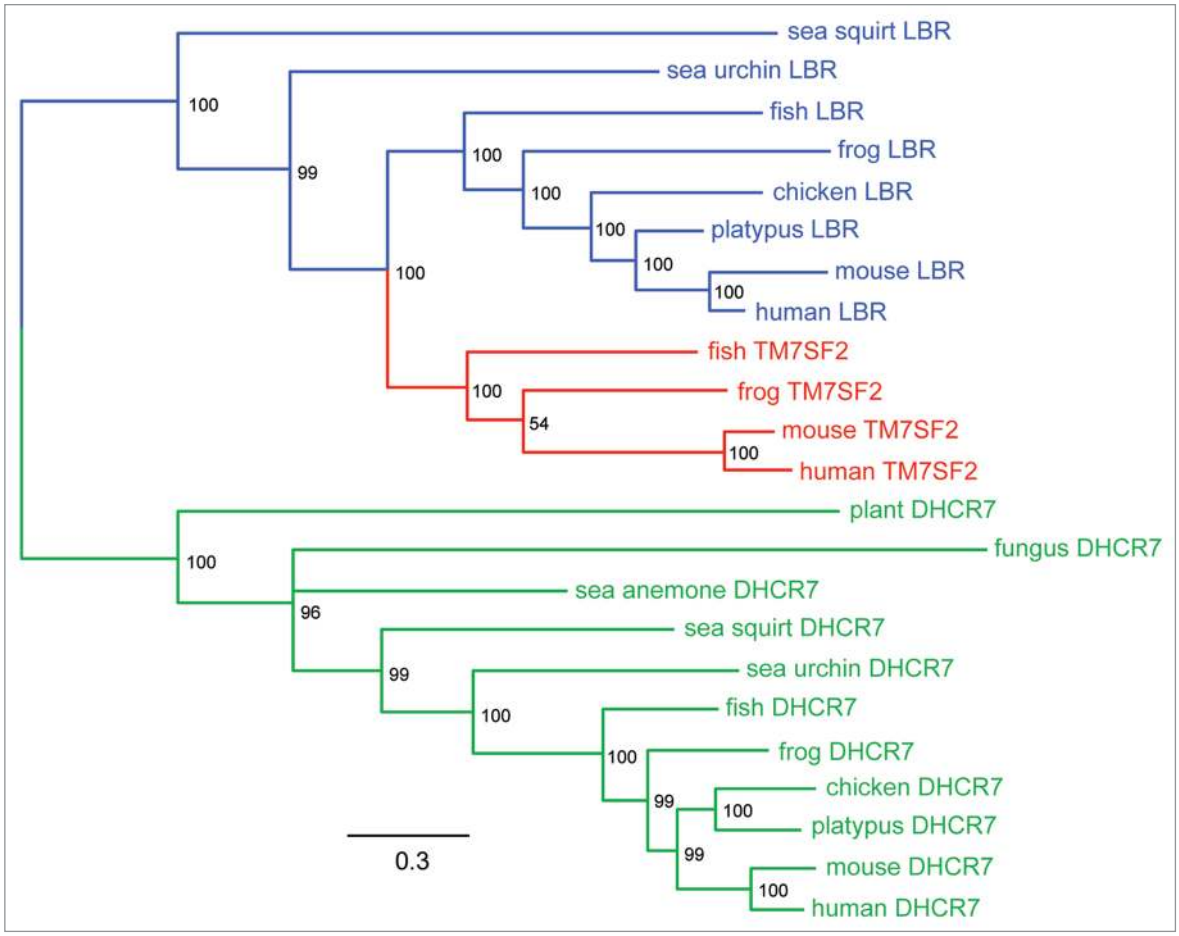

Figure 4. Bayesian phylogenetic tree Of LBR, TM7SF2 And DHCR7 From selected taxa. The taxa are: human (*Homo sapiens*); mouse (*Mus musculus*); platypus (*Ornithorhynchus anatinus*); chicken (*Gallus gallus*); frog (*Xenopus laevis*); fish (*Danio rerio*); sea urchin (*Strongylocentrotus purpuratus*); sea squirt (*Ciona intestinalis*); sea anemone (*Nematostella vectensis*); fungus (*Aspergillus oryzae*); and plant (*Arabidopsis thaliana*). Where LBR or TM7SF2 is not present in the tree it was not found in the genome of that taxon. The clades of LBR, TM7SF2 and DHCR7 are colored blue, red and green, respectively; percent posterior probability support for each node is shown; the scale bar represents changes per nucleotide position. Peptide sequences were aligned independently with Muscle[140] and T-Coffee;[141] these alignments were used with the program Combine[141] to generate a final peptide alignment that was used to align the nucleotide coding sequences. The tree was generated using MrBayes 3.1,[142] employing the general time reversible model, with rate variation modeled using a gamma distribution and allowed to vary over time (nst = 6, rates = gamma, covarion = yes). Evolution of codon third positions was modeled independently of evolution at codon first and second positions (unlink revmat, statefreq and shape). Two independent runs of 4 chains each were run for 2e6 generations and sampled every 100 generations; comparison of the parameter estimates from the two runs indicated convergence. The first 1e5 trees were discarded as "burnin" before generating the consensus tree.

Table 3. Conservation of LBR aromatic cage residues

Species	Residue number*				
	16	23	41	43	45
Human	W	Y	Y	D	T
Mouse	W	Y	Y	D	T
Platypus	W	Y	Y	D	T
Chicken	W	Y	Y	D	T
Clawed Frog	W	Y	Y	D	T
Zebra Fish	W	Y	Y	D	T
Sea Urchin	W	F	F	D	T
Sea Squirt	W	F	F	D	T

*Based on human LBR sequence; Single letter amino acid code: W, tryptophan; Y, tyrosine; F, phenylalanine; D, aspartic acid; T, threonine

ERG4_ERG24 domain is much less significant than other LBR or DHCR7 proteins. Consistent with this, the protein lacks sterol reductase activity (testing for complementation of the yeast *erg24* mutant).[128] Other dissimilarities that we note with deuterostome LBR include the absence of a Tudor domain and a reduced RS region. The authors[128] suggest that the sterol reductase activity of dLBR has been lost during evolution, in parallel with the derived inability (devolution) of insects to synthesize sterols de novo. A recent article[129] has suggested that an ortholog to dLBR can be found in the genome of *C. elegans*, which is also auxotrophic for sterols. Like dLBR, the product of this gene would lack a Tudor domain. The authors propose that both the Drosophila and *C. elegans* proteins have evolved new functions, such as involvement with intracellular protein-trafficking and/or protein folding. We note that we were unable to identify a DHCR7 ortholog

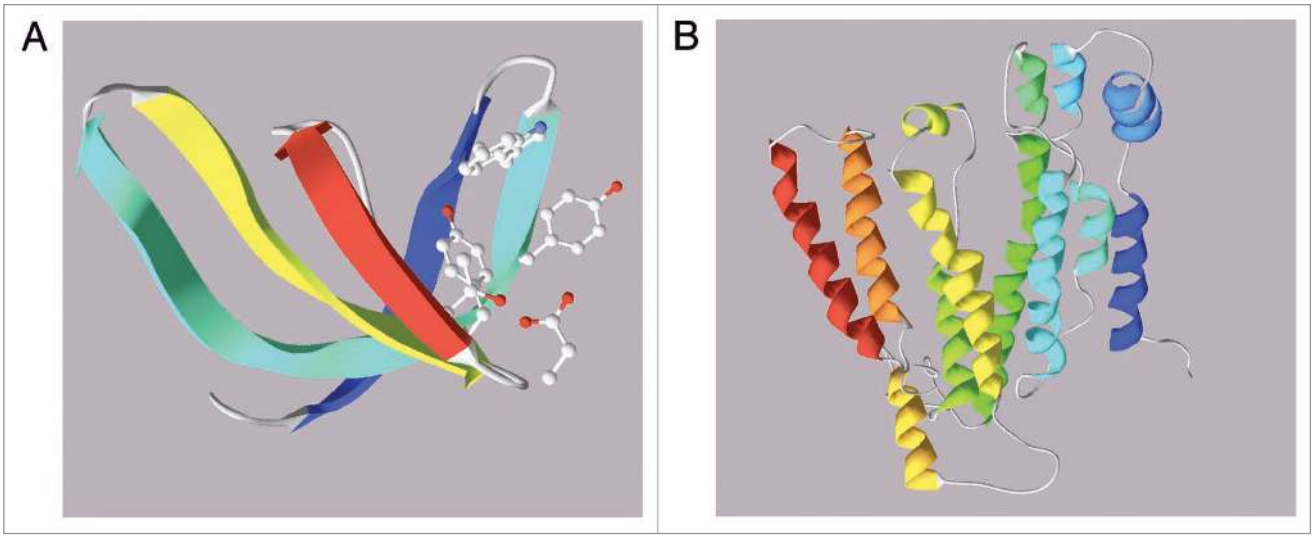

Figure 5. Structural models of human LBR, Modified From MODBASE (http://Salilab.Org/Modbase). (A) Tudor domain (residues 2–55), presenting a different view of 2dig, rotated ~90° around the vertical axis, as depicted in Figure 2A; (B) C-terminus of human LBR (residues 213–508). The homologous "template" for the structural model of the C-terminus is the crystal structure of aberrant Ba-3 cytochrome *C* oxidase from Thermus thermophilis (PDB: 1ehk). It should be mentioned that cytochrome *C* oxidase has only marginal amino acid homology (~15%) with human LBR. Color-coding of the direction of the peptide chain in both models is as described in **Figure 2**.

in the complete genome of either species, which is startling given its wide conservation in eukaryotes. The evolution of dLBR and the *C. elegans* protein may have been influenced by the loss of DHCR7; given the lack of a Tudor domain and of C14 sterol reductase activity (in dLBR) they are unlikely to have a similar function to deuterostome LBRs. Thus we suggest that referring to them as LBRs may be misleading.

Conundrums and Speculations

How should we define LBR? What is its structure within the INM? What are its functions within the INM? Which of the various putative binding partners and post-translational modifications are important for the presumed multiplicity of LBR functions? Unfortunately, the answers to these questions still evade us. Furthermore, it is likely that a clear answer to the first question (i.e., the "definition," for phylogenetic purposes) will be one of the most difficult, since it depends upon the other answers, plus a convincing evolutionary historical reconstruction.

What are some of the structural questions that need to be answered? There must be a clear identification of which ligand(s) actually bind to the LBR Tudor domain and whether this binding results in conformational and functional consequences. Our predictions that the LBR Tudor domain might have a preference for H4K20me2 acquires more significance in the light of the earlier data that methylated lysines of histone H3 and H4 are observed in immunopurified complexes with LBR.[46] We need to know whether deuterostome HP1 proteins can be documented to bind to their corresponding species LBR. Unfortunately, the putative HP1 CSD-binding motif (i.e., VxVxL in human LBR)[43] can not be found in other deuterostome LBR proteins (data not shown). Does this invalidate the binding motif? Or will it eventually be determined to be very species-specific?

The number and 3-D positioning within the INM of the TM segments of LBR must be solved. A prediction of the LBR C-terminal conformation (residues 213–508) is available from ModBase[130] (as is a model of the Tudor domain); both are shown in **Figure 5**. In addition, considerable recent progress has been made in the accurate prediction of the 3-D structure of α-helical membrane proteins, utilizing cryo-electron microscopy at a resolution sufficient to provide structural constraints combined with sophisticated computing methods.[131-133] Examples of models for α-helical membrane proteins can be found at several websites (for example, http://blanco.biomol.uci.edu/Membrane_Protein_xtal.html), but none (except the ModBase prediction) is yet available for the LBR C-terminus. Furthermore, it is not clear whether LBR forms higher quaternary structures within the INM, although this possibility has been suggested.[46] Ideally, we would like to see LBR expressed and embedded into synthetic membranes of varying composition, to determine whether the presence (or absence) of sterols influences the tertiary and quaternary structures, and the sterol reductase activity.

What are the functional issues to be addressed? We need to know whether the N-terminus of LBR interacts with repressed heterochromatin, active euchromatin or intranuclear RNA-containing bodies. Current evidence favors the first option. It would be useful to see whether mutations in the N-terminus influence the higher order interphase nuclear architecture. Presently, there is only suggestive evidence that the absence of LBR might influence expression of some genes. Mouse homozygous *ic/ic* EPRO cells reveal a surprising increase in lamin A/C expression, besides the ovoid nuclei and redistributed heterochromatin.[85] We can only speculate that the presence of sufficient LBR during granulopoiesis may function directly (or indirectly) to silence the lamin A/C gene. Possible recruitment of the lamin A/C gene into the perinuclear heterochromatin could be explored using in situ hybridization technology. It

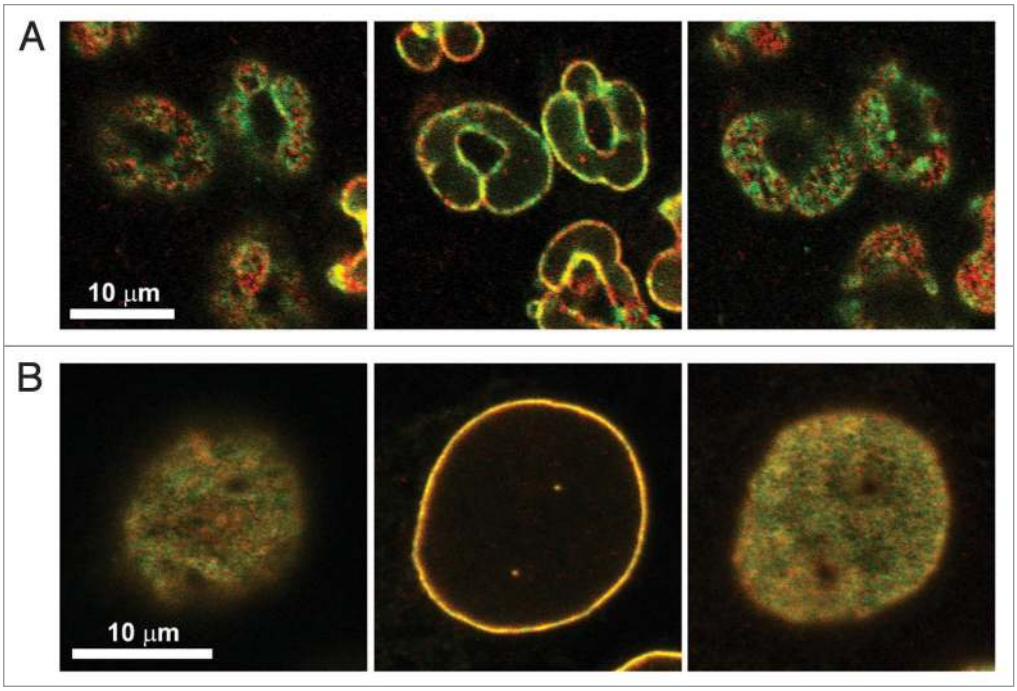

Figure 6. Imperfect co-localization of LBR and lamin B as studied by immunofluorescent staining. (A) Granulocytic MPRO nuclei: The ring-shaped granulocyte nuclei were stained with anti-LBR (Cy3, red) and anti-lamin B (FITC, green), see for details.[139,143] The mid-section is the middle image; left and right images are the top and bottom surfaces of the NE. (B) U2OS: The ovoid nucleus was stained with anti-LBR (Alexa 488, green) and anti-lamin B1 (Cy3, red). Note the apparently good co-localization of the two colors in the mid-section view; whereas, the top (left) and bottom (right) nuclear surfaces exhibit islands of red and of green.

is worth pointing out that the adult human tissue with the highest level of LBR mRNA expression is bone marrow.[134]

There are tantalizing indications of a "connection" between lamin A/C and LBR. Lamin A has been identified in a detergent solubilized "LBR complex" isolated by immunoprecipitation.[18] In addition, a more recent study of a lamin A mutation in a specific patient with autosomal-dominant Emery-Dreifuss muscular dystrophy,[135] indicated partial loss of LBR from the NE to the ER. The authors speculated that some unknown nuclear protein might be mediating the interaction between lamin A and LBR, and that this particular lamin A mutation is unable to bind to the unknown protein. To our knowledge, there have been no other searches for this postulated bridging protein.

It is not clear what evolutionary pressures selected for concentrating a C-14 sterol reductase (LBR) within the INM, when all the other cholesterol biosynthetic enzymes (post-squalene) reside within the ER. (A small amount of LBR can be frequently observed within the ER). We[85] and others[24,51] have speculated that LBR plays a special role in NE membrane formation, possibly most crucially during post-mitotic NE reformation.[53,70,71] However, there must be functional redundancy, since homozygous *ic* EPRO cells are deficient in LBR but can still divide (and differentiate) in vitro.[85,87]

Our speculation that LBR is involved with NE membrane growth is based upon our studies with granulocytic cells differentiation in vitro and in vivo: (1) a positive correlation was observed between increased LBR levels in HL-60 cells, appearance of nuclear lobulation and formation of ELCS;[81,82,89] (2) a correlation between LBR mutations resulting in a deficiency of LBR and hypolobulation of granulocyte nuclei in human PHA and mouse ichthyosis (*ic*);[79,84,86] (3) an estimated reduced NE surface area in EPRO cells derived from homozygous *ic* bone marrow cells.[85] Overexpression of LBR in yeast[24] and HeLa[51] is certainly consistent with our speculation, but overexpression of membrane-associated protein may be fraught with artifactual issues.[136]

When a membrane resident protein (cytochrome b5) was expressed (with or without GFP) in COS-7 cells, an extensive formation of stacked "organized smooth ER (OSER)" membranes was observed in the cytoplasm and adjacent to the NE.[136] The authors demonstrated that the protein minimal requirement for OSER formation was a transmembrane region coupled to a cytoplasmic portion which is capable of "homotypic" interactions (GFP, being sufficient; but a nondimerizing GFP, not sufficient). Later studies extended these observations to lamins and stressed the importance of isoprenylation of the cysteine in the C-terminal CaaX motif.[137,138] In the first study,[137] GFP-lamins B1 and B2 were expressed in Xenopus oocytes and extensive stacked membrane arrays were observed within the nucleus. Similar intranuclear membrane stacks were observed in HeLa cells transfected with a plasmid containing an NLS-GFP-CaaX construct. In the second study,[138] Xenopus A6 cells were transfected with a variety of lamins from different species. Moderate levels of expression of lamins A and B2 exhibited nuclear lobulation, with essentially no intranuclear membrane arrays; lamin C, without the CaaX motif did not induce lobulation

or intranuclear membranes. High levels of expression of GFP-lamins (not including lamin C) lead to extensive intranuclear membranes. Mutation of the cysteine to a serine in lamin B2 CaaX prevented the membrane growth. The importance of isoprenylation of cysteine in the CaaX motif is further underscored by the observation that a Drosophila protein that regulates NE size and shape ("Kugelkern") contain an NLS and a C-terminal CaaX motif, and is a candidate for farnesylation.[138] No convincing mechanism has been demonstrated connecting integration (or binding) of a protein into the NE or ER membranes with membrane growth. Current models often include the suggestion that structural changes in the membrane due to protein insertion, trigger lipid synthesis in a manner analogous to the feedback control of cholesterol biosynthesis. Studies are clearly required which would examine the quantitative and qualitative changes in cellular membranes during the induction of OSER or ELCS, and of nuclear lobulation. It is also interesting to speculate that the LBR induction of NE membrane changes might be a direct effect, or an indirect effect, due to its possible cysteinyl methyl transferase (ICMT) activity operating upon the lamin CaaX motif.

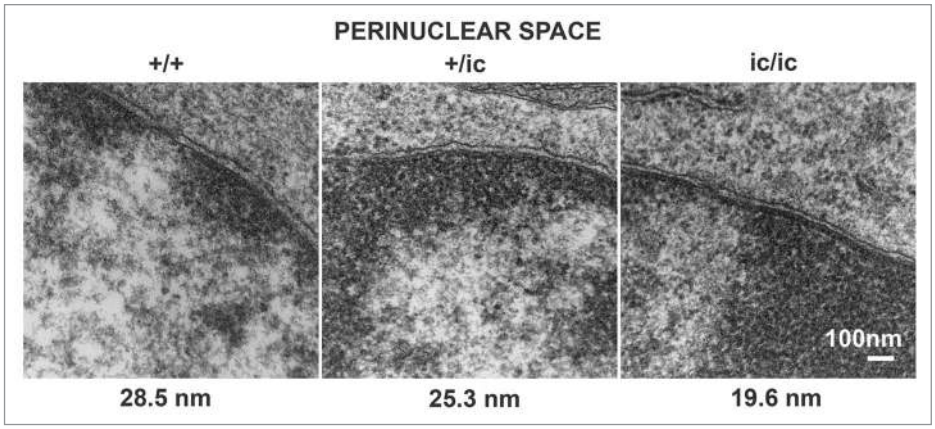

Figure 7. Thin-section electron micrographs of undifferentiated EPRO cells with measurements of the perinuclear cisternal width. Images for three genotypes are shown: +/+; +/*ic*; *ic/ic*. In each case, the average distance across the cisternae was determined, based on a total of >170 measurements. Also notice the presence of a ~20 nm wide layer of chromatin, immediately adjacent to the NE, regardless of genotype. Measurements were also performed on thin-sections of granulocytic EPRO cells, with virtually identical results (data not shown).

During our laboratory's explorations of LBR and nuclear shape, we have encountered several puzzling microscope images, which are documented here; hopefully, they will stimulate further research: (1) Confocal images (**Fig. 6**) of mouse granulocytic MPRO cells[139] and human U2OS cells immunostained for LBR and lamin B revealed that, although both are localized in the NE, optical slices from the top and bottom of the nuclei revealed imperfect co-localization! This was not an expected result, given the presumption that LBR binds lamin B. It is important to repeat this experiment with in vivo fluorescent staining. (2) Thin-section electron microscopy of undifferentiated (**Fig. 7**) and granulocytic EPRO cells[87] revealed, quite surprisingly, that the distance between ONM and INM significantly decreased, progressing from (+/+)>(+/*ic*)>(*ic/ic*). Does this mean that the C-terminus of LBR points into the lumen, contributing to a protein bridge that separates the two NE membranes? (3) The same electron microscope images of EPRO cells (**Fig. 7**) showed that, despite the significant movement of heterochromatin away from the NE in granulocytic (*ic/ic*) cells, there remains a layer of chromatin just under the NE? Clearly, some other protein(s) are binding this chromatin to the

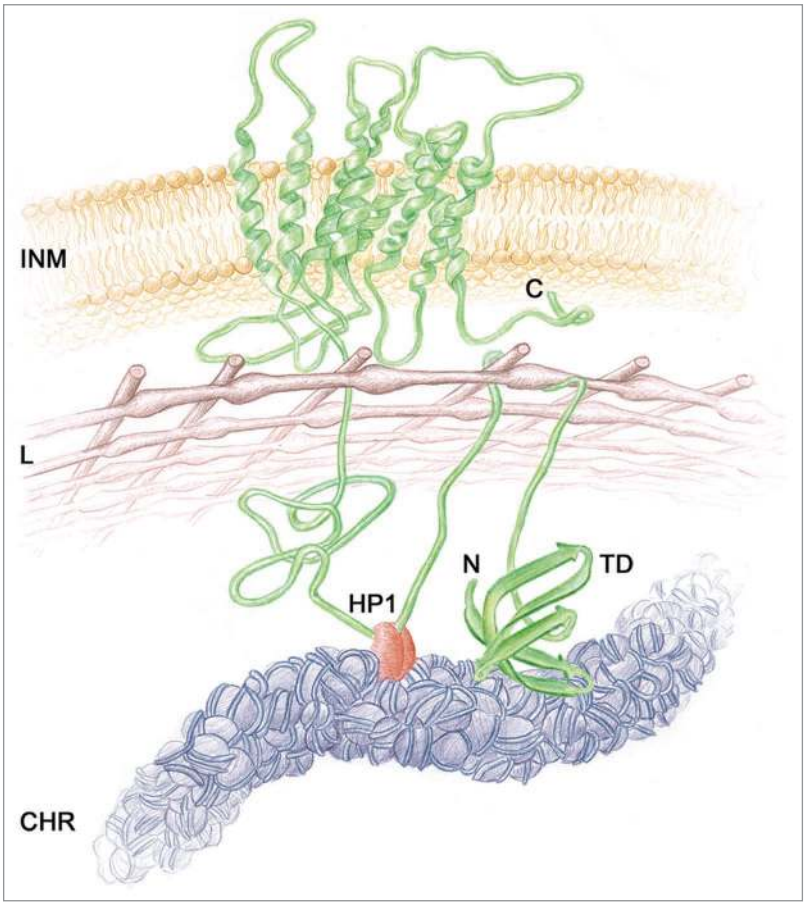

Figure 8. Cartoon representation of LBR embedded within the nuclear envelope. Landmarks: INM, inner nuclear membrane (yellow); N and C, termini of LBR (green); L, lamin meshwork (brown); TD, Tudor domain; HP1, heterochromatin protein 1 (red); CHR, peripheral heterochromatin with nucleosomes (blue). For illustrative purposes, the dimensions are distorted, with LBR drawn at the highest magnification. Other structures are also not drawn to scale in this cartoon; for currently accepted dimensions, the INM is ~5 nm thick; L, ~15 nm thick; CHR, ~20–30 nm diameter.

NE. What are those proteins? Is this a unique category of chromatin or DNA?

Our Current View

We think that LBR has at least three important functions for the cell: (1) contributing to post-mitotic nuclear reformation; (2) contributing to interphase NE growth; (3) contributing to compartmentalization of heterochromatin within the interphase nucleus. LBR may play other roles, such as a "back-up" enzyme for sterol biosynthesis or for lamin isoprenylation, under certain conditions and in specific cells. With respect to post-mitotic nuclear reformation, we suggest that the early binding of LBR to decondensing mitotic chromosomes is mediated by LBR Tudor domain-H4K20me2, by LBR-HP1-H3K9me3 and by LBR basic RS region-DNA interactions. The consequence of these interactions is to attach the post-mitotic ER to the heterochromatin. Later interactions with lamin B might be mediated by presently unknown binding sites on the LBR N-terminus and by putative ICMT functionality interacting with isoprenylated cysteine derived from the lamin CaaX. Interphase NE growth, as exemplified by NE expansion during S phase or ELCS formation in granulocytic HL-60 cells, may be related to both the sterol reductase and putative ICMT functions as the levels of NE bound LBR increase (a point not yet studied in S phase cells). Compartmentalization might be accomplished in the interphase nucleus by LBR-heterochromatin interactions, mentioned earlier, bringing pericentric and transcriptionally-inactive regions adjacent to the NE. **Figure 8** presents a cartoon of LBR embedded within an interphase NE. This fanciful drawing (not to scale) displays the C-terminus with multiple TM segments spanning the INM, regions of the N-terminus threading through and interacting with the lamina meshwork, and the Tudor and HP1 binding domains forming attachments with the peripheral heterochromatin. We believe that when LBR is fully understood, it will present a fascinating example of a protein that exists in two worlds (i.e., hydrophilic and hydrophobic) with multiple tasks in the NE, bridging and communicating structural and functional information.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/OlinsNUC1-1-Sup.pdf

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