Requirement for Lamin B Receptor and Its Regulation by Importin β and Phosphorylation in Nuclear Envelope Assembly during Mitotic Exit

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Lamin B receptor (LBR), a lamin and lamin B-binding protein in the inner nuclear membrane, has been proposed to target the membrane precursor vesicles to chromatin mediated by importin β during the nuclear envelope (NE) assembly. However, the mechanisms for the binding of LBR with importin β and the membrane targeting by LBR in NE assembly remain largely unknown. In this report, we show that the amino acids (aa) 69–90 of LBR sequences are required to bind with importin β at aa 45–462, and the binding is essential for the NE membrane precursor vesicle targeting to the chromatin during the NE assembly at the end of mitosis. We also show that this binding is cell cycle-regulated and dependent on the phosphorylation of LBR Ser-71 by p34
tubulin kinase. RNAi knockdown of LBR causes the NE assembly failure and abnormal chromatin decondensation of the daughter cell nuclei, leading to the daughter cell death at early G1 phase by apoptosis. Perturbation of the interaction of LBR with importin β by deleting the LBR N-terminal spanning region or aa 69–73 also induces the NE assembly failure, the abnormal chromatin decondensation, and the daughter cell death. The first transmembrane domain of LBR promotes the NE production and expansion, because overexpressing this domain is sufficient to induce membrane overproduction of the NE. Thus, these results demonstrate that LBR targets the membrane precursor vesicles to chromatin by interacting with importin β in a LBR phosphorylation-dependent manner during the NE assembly at the end of mitosis and that the first transmembrane domain of LBR promotes the LBR-bearing membrane production and the NE expansion in interphase.

Nucleus, the largest organelle that contains the genome of the eukaryotic organisms, is surrounded by a continuous nuclear envelope (NE) composed of a pair of inner and outer nuclear membranes, studded by numerous nuclear pore complexes. Through the nuclear pore complexes, the NE controls the flow of molecules between the nucleus and the cytoplasm in a tightly regulated manner (1–5). The NE is highly dynamic during the cell cycle. It disassembles into membranous vesicles or tubules and disperses into the cytoplasm at the onset of pro-metaphase and reassembles around the newly replicated chromosomes at the end of mitosis using their previously disassembled components (2, 4, 6). Recently, it was shown that Ran, a small Ras-like nuclear GTPase, and its binding proteins regulate the NE assembly process (7–13). Ran exists in GTP- and GDP-bound states that interact differently with effectors. Conversion between these two states and the assembly or disassembly of the effector complexes requires the interaction of regulatory proteins, the nucleus-based guanine nucleotide exchange factor RCC1 and the cytoplasm-localized GTPase-activating protein RanGAP1 (6, 14). The compartmentalization of the regulators produces a high concentration gradient of Ran-GTP in the nucleus and Ran-GDP in the cytoplasm. In mitosis, after the NE breakdown, the nucleoplasm and the cytoplasm are mixed; Ran diffuses into the cytoplasm with many other soluble nuclear proteins; RCC1 dynamically binds with the mitotic chromosomes and promotes the establishment of a concentration gradient of Ran-GTP/GDP where it is found at highest near the chromosomes and lowest at the centrosomes. During the NE assembly at the end of mitosis, Ran rebinds the chromatin and regulates the NE assembly, which depends on its GTPase cycle and its regulators (6–11, 14).

Although Ran and its regulators have been shown crucial for the NE assembly, the mechanism for the NE precursor vesicle recruitment to chromatin and the downstream effectors remain poorly understood (8–11, 15). Importin β interacts with the FXFG domain of nucleoporins and targeting them to the chromatin (8, 13). Importin β likely works with other Ran-binding proteins to regulate the nuclear pore complexes and NE formation through interacting with the FXFG domain nucleoporins and targeting them to the chromatin (8, 13). For example, importin β depletion in Xenopus egg extracts blocks the NE assembly, whereas adding back exogenous importin β rescues the NE formation in an appropriate concentration-dependent manner (8). Moreover, importin β-coated beads alone could induce the NE assembly in Xenopus egg extracts under the regulation of Ran (8). In Caenorhabditis elegans embryos, importin β knockdown by RNAi also blocks the NE assembly.
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(18). We have recently reported that importin β plays a crucial role in the recruitment of the lamin B receptor (LBR)-bearing NE precursor membrane vesicles to the chromatin surface and the NE assembly through a direct interaction with the N-terminal spanning domain of LBR at amino acids (aa) 45–90 (15). Furthermore, these activities were carried out in a Ran-sensitive and importin α-independent manner (15).

LBR appears to be a central player in targeting nuclear membranes to the chromatin (4, 15). LBR is a polytopic inner nuclear envelope protein with eight predicted hydrophobic transmembrane domains (76). The hydrophilic N-terminal spanning region of LBR is composed of ~200 aa and exposed to the nucleoplasm, where it interacts with chromosome/chromatin proteins such as B-type lamins, HA95, and heterochromatin protein 1 (HP1) (19–30). The interaction of LBR with HP1 may not be direct, but is mediated through the core histones H3/H4 (31). The N-terminal spanning region of LBR contains a conserved arginine/serine (RS)-rich region, which may be phosphorylated on some serine and threonine residues by multiple RS kinases in a cell-cycle-dependent manner (32, 33). LBR phosphorylation appears to regulate its binding to chromatin proteins (32–37). The C terminus of LBR is a short hydrophilic spanning region facing to the nucleoplasm with an unknown function (38). The large middle part from aa 211–615 of the LBR houses the 8 transmembrane domains. The first transmembrane domain, but not the others, is critical for confining this protein to the inner nuclear membrane (39). During the NE assembly at the end of mitosis, it is found that LBR plays a central role in targeting the NE precursor membrane vesicles to the chromatin mediated by importins. Overexpression of LBR in yeast cells induces over-generation of membrane and formation of membrane stacks in the cytoplasm (40). In sea urchins, LBR is required for targeting the LBR-bearing membrane vesicles to the chromatin and the NE assembly during male pronuclear formation (41). In culture cells, LBR fused with the green fluorescent protein (GFP) has been used to follow the dynamics of the NE assembly and the mechanism investigation of the NE membrane precursor targeting to the chromatin (42, 43). In a more recent study we found that, during the NE reassembly, the targeting of the NE membrane precursor vesicles to the chromatin is mediated by the interaction of the N-terminal spanning region with importin β (15). Once meeting with Ran-GTP on the surface of the chromatin generated by RCC1, importin β preferentially binds with Ran-GTP and releases the LBR-bearing vesicles to assemble the NE. With the hydrolysis of the GTP molecule of Ran, the membrane vesicles fuse to form the bi-layered NE and importin β leaves Ran for next cycle (6, 15). However, how the interaction between importin β and LBR is regulated and why overexpression of LBR causes over-generation of the membrane remain unclear.

In this work, we report that the targeting of the LBR-bearing NE precursor membrane vesicles to the chromatin during the NE assembly is mediated by importin β through interaction of its aa 45–462 with the aa 69–90 of LBR. LBR knockdown causes the NE assembly failure and the cell death in early G1 phase. We also show that the phosphorylation status of LBR serine 71 regulates the interaction between LBR and importin β. Deletion of the N-terminal spanning domain or aa 69–73 of LBR reduces the binding strength of LBR to the chromatin in interphase, increases the lateral movement potential of LBR, prevents successful targeting of the LBR-bearing NE precursor membrane vesicles to the chromatin at the end of mitosis, and causes the cell death of the daughter cells in next early G1 phase. We also find that the first transmembrane domain of LBR is responsible for the membrane production. Together, our results demonstrate that LBR performs a crucial role in recruiting the LBR-bearing NE precursor membrane vesicles to the chromatin during the NE assembly mediated by importin β in a phosphorylation-dependent manner in mitosis and that the first transmembrane domain of LBR is responsible for the membrane production and the targeting of this protein to the NE during the nuclear growth and the NE expansion in interphase.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Wild-type Xenopus LBR (NCBI Y17842) was cloned from a cDNA library of Xenopus oocytes (Clontech) and inserted into pEGFP-C2 vector (Invitrogen) at the EcoRI and SalI restriction sites. GFP-xLBR1–210 was constructed by inserting the cDNA encoding aa 1–210 into the EcoRI/SalI sites of pEGFP-C2. GFP-xLBR1–380, GFP-xLBR1–462, and GFP-xLBR1–531 were subcloned into the PstI and SalI sites of pEGFP-C2 (31). The N-terminal spanning domain or aa 69–73 of LBR reduces the binding strength of LBR to the chromatin in interphase, increases the lateral movement potential of LBR, prevents successful targeting of the LBR-bearing NE precursor membrane vesicles to the chromatin at the end of mitosis, and causes the cell death of the daughter cells in next early G1 phase. We also find that the first transmembrane domain of LBR is responsible for the membrane production. Together, our results demonstrate that LBR performs a crucial role in recruiting the LBR-bearing NE precursor membrane vesicles to the chromatin during the NE assembly mediated by importin β in a phosphorylation-dependent manner in mitosis and that the first transmembrane domain of LBR is responsible for the membrane production and the targeting of this protein to the NE during the nuclear growth and the NE expansion in interphase.

Protein Expression and Purification—Escherichia coli strain BL21 (pLys) was transformed with His-xLBR1–210, His-xLBR1–210–S71A, His-xLBR1–210–S71D, His-xLBR1–380, His-xLBR1–462, GST-importin β1–462, GST-importin β1–71–462, GST-importin β1–71–380, and GST-importin β1–380. To produce the recombinant His-tagged LBR proteins, the bacterial cells were grown to an A600 of ~1.0. Isopropyl-β-D-thiogalactopyranoside was then added to a final concentration of 0.1 mM, and the cells were incubated at 30 °C for more than 5 h to induce protein expression. To produce recombinant GST-tagged importin β proteins, the bacterial cell cultures were grown to an A600 of ~0.5. Isopropyl-beta-D-thiogalactopyranoside was then added at a final concentration of 0.1 mM, and the cultures were incubated at 17 °C for >6 h to induce the protein expression. The bacterial cells were pelleted by centrifugation at 6,000 × g for 10 min at 4 °C. Proteins were purified with either Talon-Resin (BD Bioscience) or Glutathione-Sepharose 4B (Pharmacia Biotech Inc.) following the manufacturer’s instructions.
Cell Culture, Transfection, Synchronization, and RNAi—HeLa cells were grown in DMEM medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma), on 35-mm diameter Petri dishes at 37 °C in a 5% CO₂ atmosphere and transfected using the calcium precipitation method as described previously (44). Briefly, the precipitated plasmid DNA was left with the cells in the Petri dishes for 6 h. Cells were then washed with PBS and grown for another 24–48 h before direct observation and fixation for immunofluorescence microscopy.

For cell synchronization, HeLa cells were grown in DMEM, treated in thymidine (2.5 mM) for 20 h, released in fresh medium for 4 h, and then treated with nocodazole (50 nM) for 12 h. Almost all the cells were blocked at metaphase.

For RNAi, HeLa cells were transfected with LBR-HSS105976, LBR-HSS105977, and LBR-HSS105978 by Lipofectamine 2000. Samples were assayed at 72 h after transfection.

Co-immunoprecipitation and GST Fusion Protein Pulldown Assays—HeLa cells were washed once with cold PBS and lysed in cell lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EGTA, 0.5 mM EDTA, 0.5% Nonidet P-40, 5 mM NaF, 1 mM Na3VO4, 1 mM PMFS, 10 g/ml aproamin, and 5 g/ml Pepstatin A) for 20 min on ice and centrifuged at 13,000 rpm for 15 min to obtain clear cell lysates.

For co-immunoprecipitation assays, the clarified cell lysates were incubated with indicated antibodies for 60 min on ice. Then 15 μl of protein A- or G-Sepharose (75% slurry) was added, and the mixtures were rotated for 2 h at 4 °C. The beads were washed three times with lysis buffer and harvested by brief centrifugation and finally suspended in gel sample buffer.

For GST pulldown assay, 5 μg of soluble GST or GST-fused proteins bound to 15 μl of Glutathione-Sepharose beads (75% slurry) were incubated with lysates from HeLa cells for 3 h at 4 °C. The beads were washed five times with lysis buffer and harvested by brief centrifugation and finally suspended in gel sample buffer. The beads-bound proteins were separated on 10% SDS-PAGE gels and analyzed by immunoblotting (IB) with the indicated antibodies.

Gel Electrophoresis and IB—After being separated on 10% SDS-PAGE gels, the protein samples were transferred onto nitrocellulose filters in the transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 1 h at 100 V. The filters were blocked in TTBS (20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.3% Tween 20) containing 5% nonfat milk for 1 h at room temperature and probed with anti-GFP monoclonal antibody (Santa Cruz Biotechnology, diluted 1:1000 in TTBS with 5% nonfat milk) or probed with anti-importin β monoclonal antibody (BD Transduction, diluted 1:1000 in TTBS with 5% nonfat milk) overnight at 4 °C. The filters were then washed three times and blocked again for 30 min in TTBS containing 5% nonfat milk and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson, diluted 1:1000 in TTBS with 5% nonfat milk) for 1 h at room temperature. After a thorough wash in TTBS, the filters were developed for visualization by enhanced chemiluminescence (Sigma) and x-ray films.

Immunofluorescence Microscopy—Transfected cells were grown to 60% confluency on 35-mm diameter Petri dishes, washed three times with PBS, and fixed at room temperature in 4% paraformaldehyde for 15 min. The cells were then incubated with primary antibodies diluted in PBS containing 3% BSA for 1 h at room temperature or overnight at 4 °C. The antibody against cleaved caspase 3 was from Cell Signaling Technology and diluted at 1:1000. The antibody against hLBR was generated by injecting rabbits and diluted 1:500. The cells were then washed five times in PBS and incubated with secondary antibodies diluted in PBS containing 3% BSA (TRITC-goat anti-rabbit Ig (DAKO), diluted 1:200) at room temperature for 45 min to 1 h at room temperature. The cells were then washed five times in PBS and mounted in Mowiol (Sigma) containing DAPI. Samples were viewed under a Zeiss immunofluorescence microscope 200M equipped with a 63× objective. Images were captured using a cooled charged-coupled device AxioCamMRm camera.

Live Imaging—HeLa cells grown in a glass-bottomed Petri dish in a temperature-controlled chamber at 37 °C with 5% CO₂ were transfected with GFP-xLBR1–210/211 (as control), GFP-xLBR1–621/621A69–73, or full-length GFP-xLBR1–621 (as control). The images of the cells expressing the GFP fusion protein were acquired every 5 min by a cooled charged-coupled device AxioCamMRm camera on an Axiovert 200M microscope. The Axiovert software was used to collect and process the data.

Preparation of Xenopus Egg Extract and Demembraned Xenopus Sperm Chromatin and the NE Assembly in Vitro—Low speed Xenopus egg extracts were prepared as described previously (45–47). The extracts were then frozen and stored in aliquots in liquid nitrogen until use.

Sperms were released from testes by gently squeezing in nuclear isolation buffer (NIB, 15 mM NaCl, 60 mM KCl, 15 mM Tris, pH 7.5, 1 mM dithiothreitol, 0.5 mM spermine, and 0.25 mM sucrose) and centrifuged at 100 × g for 1 min at 4 °C to remove somatic tissues. Sperm-containing supernatant was centrifuged at 1,500 × g for 10 min at 4 °C. The pellet was re-suspended in NIB and incubated in the presence of 0.05% lysolce- thin for 8–10 min at 23 °C. The reaction was stopped by adding 3 volumes of cold NIB plus 3% BSA. After washing for three times with NIB, the demembranated sperm chromatin were stored in aliquots in liquid nitrogen at 10⁹/ml.

Low speed egg extract was mixed with an ATP-regenerating and the demembranated sperm chromatin. The reaction system contained 20 μl of low speed egg extract, 1 μl of ATP-regenerating system, and ~2000/μl demembranated sperm chromatin. The proteins His-xLBR1–210, His-xLBR1–210–S71A, His-xLBR1–210–S71D, or EB buffer (extract buffer) alone was added into the reaction system to a final concentration of 10 μM. The samples were removed after incubation at 23 °C for the time indicated and stained on a slide with 3,3-dihexyloxyacarbocyanine without fixation. The samples were immediately viewed under a Zeiss 200M immunofluorescence microscope equipped with a 63× objective. Images were captured using a cooled charged-coupled device AxioCamMRm camera.

FRAP Analysis—Fluorescence recovery after photobleaching (FRAP) was performed with a Leica confocal microscope using a 488-nm line of a 400-milliwatt Kr/Ar laser in conjunction with a 100× objective for optimum resolution. For quantitative essay, the framed areas were photobleached at full laser power (100% power and 100% transmission) for 2 s, and recovery of
the fluorescence was monitored by scanning the whole cell at low laser power (19% power and 19% transmission) in a 20-s intervals. The scanning laser intensity did not significantly photobleach the specimen over the time course of the experiment.

**MS Analysis of the Phosphorylated Proteins**—2 μg of His-hLBR 1–210 was incubated with cyclin B/Cdc2 (New England Biolabs) in 50 mM Tris, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, 5 mM DTT, 100 mM ATP, for 30 min at 30 °C. The reactions were subjected to SDS-PAGE, and the gels were processed for MS analysis. The targeted gel band was cut and destained by 25 mM NH₄HCO₃ in 50% acetonitrile. The gel band was dried in 100% acetonitrile with a linear gradient from 2% to 35% buffer B (100% acetonitrile 0.1% acetic acid) at a flow rate of 500 nl/min over 40 min using a 150-μm inner diameter × 150-mm reverse-phase C18 columns (Microtech Scientific). The MS was operated in data-dependent MS/MS mode in each full MS scan was followed by five MS/MS scans where the five most abundant peptide ions were selected for collision-induced dissociation using a normalized collision energy of 35% in the ion trap. Survey MS scans were acquired in the Orbitrap analyzer with resolution of 60,000 (m/z 400) in the positive ion mode. Raw MS files were processed with Proteome Discoverer 1.0 (ThermoFisher Scientific), and searched against the IPI-human (version 3.55 European Bioinformatics Institute) containing both forward and reversed protein sequences. Initial maximum precursor and fragment mass deviations were set to 7 ppm and 0.5 Da, respectively. The search parameters included variable modifications for oxidation of methionine, and phosphorylation of serine, threonine, and tyrosine and variable modifications for cysteine carbamidomethylation. The maximum peptide false discovery rate was set to 0.01.

**RESULTS**

**LBR Interacts with Importin β at Mitosis and They Become Disassociated at the End of Mitosis**—The NE assembly is one of the key steps in generating daughter nuclei during the cell division in eukaryotic cells. LBR has been shown to play a vital role in targeting the membrane vesicles to the chromatin during the NE assembly at the end of mitosis (15). Although it is known that the targeting of the membrane to the chromatin by the LBR indicates that LBR interacts with importin β and LBR is dynamic during mitosis. We synchronized them to interphase or mitosis and performed co-immunoprecipitation experiments using an anti-GFP antibody, followed by Western blots using the anti-importin β antibody to reveal the interaction of GFP-LBR with importin β. The results showed that the endogenous importin β co-precipitated with both GFP-xLBR1–210 and GFP-hLBR1–210 from the mitotic but not the interphase cell lysates (Fig. 1B). This result indicates that LBR interacts with importin β mostly during mitosis.

![Figure 1](image_url)

**FIGURE 1.** LBR1–210 interacts with importin β at mitosis and dissociates at the end of mitosis. A, HeLa cells, transiently transfected with GFP-xLBR1–210, were co-stained with anti-importin β and DAPI for DNA. Note that this truncate LBR localizes to the nucleus in interphase and moves to mitotic spindle and other cytoplasmic place where it co-localizes with importin β in mitosis. B, co-immunoprecipitation of importin β with GFP-xLBR1–210. The clarified xLBR1–210, expressing cells in both interphase and mitosis were lysed and incubated with a rabbit anti-GFP or rabbit-IgG (as control) antibody for 60 min on ice. Then 15 μl of protein A- or G-Sepharose (75% slurry) were added, and the mixtures were rotated for 2 h at 4 °C. The beads were washed three times with lysis buffer and harvested by brief centrifugation and finally suspended in gel sample buffer. The protein samples were separated by SDS-PAGE gel and processed for Western blot with anti-importin β or anti-GFP antibody. Note that the binding of the truncate GFP-xLBR1–210 with importin β occurred only in mitosis. C, HeLa cells were arrested by double thymidine block/release to various cell cycle phases and lysed to generate time-course samples, and the time at the metaphase onset is referred to as 0 min. The cell lysates were processed for GST pulldown assay between GFP-xLBR1–210 and GST-importin βS–622 using an anti-GFP antibody, followed by Western blot. D, HeLa cells were arrested by thymidine/nocodazole block at metaphase and released. Cell lysates were prepared at the time indicated after release from metaphase and GST pulldown assay between GFP-xLBR1–210 and GST-importin βS–622 was performed using anti-GFP antibody. The lysate GFP-LBR1–210 in GST pull-down assay was used as control. Note that the interaction of GFP-LBR1–210 became weaker along with the release from metaphase.
in corresponding cell lysates, followed by Western blot using an anti-GFP antibody. The result showed that a weak interaction between importin β and the LBR started during the S and G2 phases, then became stronger as the cells entered mitosis, reached peak at 90 min after entry into mitosis, and declined until it disappeared during the G1 phase (Fig. 1C). We also performed similar pulldown assays using metaphtase-arrested -released cells expressing GFP-xLBR1–210 and GST-importin β45–462. Consistent with the previous observations, we found that a strong interaction between LBR and importin β occurred within 30 min after metaphase onset. Following that, the interaction attenuated till it became undetectable after 50 min when most of the cells entered the G1 phase (Fig. 1D). Together, these results demonstrate that the strong binding of LBR with importin β is mitosis-specific and that they dissociate at the end of mitosis.

aa 69–90 Are Required for the Binding of LBR with aa 45–462 of Importin β and the Binding Is Regulated by Phosphorylation of LBR at Ser-71—Next, we decided to investigate the binding sites on both LBR and importin β. First, we constructed and purified a series of truncated importin β proteins fused with GST: GST-importin β1–187, GST-importin β1–462, GST-importin β1–380, GST-importin β45–462, GST-importin β71–462, GST-importin β73–380, and GST-importin β73–380 (Fig. 2A). Then we performed pulldown assays using these GST-importin β truncate proteins and the lysate of mitotic HeLa cells transfected with GFP-xLBR1–210 to determine the interaction between importin β and LBR. Our results showed that the binding affinity of GST-importin β1–462, GST-importin β45–462, or GST-importin β73–462 with LBR was as the same as that of full-length importin β, whereas the binding affinity of other truncated importin β proteins with LBR was not detectable (Fig. 2B). These results indicate that the aa 45–462 region of importin β is the minimal length required for binding with LBR (Fig. 2A and B).

It has been reported that there is a conserved serine and arginine (SR)-rich sequence in the LBR N-terminal spanning region, which contains a number of potential phosphorylation sites (Fig. 2C) (25). To reveal the binding sites on LBR for importin β, we constructed GFP-xLBR53–210, GFP-xLBR66–210, GFP-xLBR73–210, GFP-xLBR73–210, GFP-xLBR73–210, and GFP-xLBR73–210 (aa 69–73-deleted GFP-xLBR1–210), and GFP-xLBR1–210A73–90 (aa 73–90-deleted GFP-xLBR1–210) based on SR-rich sequence features. Then we expressed these truncated proteins in HeLa cells and carried out pulldown assays to detect the interaction between importin β and these truncate proteins. The result showed that both GFP-xLBR53–210 and xLBR1–210 bound with importin β efficiently, whereas GFP-xLBR66–210 showed relatively weak affinity with importin β. When the SR-rich sequence was disrupted or deleted, as in truncates GFP-xLBR1–210Δ69–73, GFP-xLBR73–210, GFP-xLBR73–210, GFP-xLBR73–210, or GFP-xLBR90–210, these truncate proteins completely lost their binding ability with importin β (Fig. 2D). This result indicates that the SR-rich sequence is crucial for the interaction of LBR with importin β. As it has been reported that serine 71 of LBR is phosphorylated by the mitotic kinase CDK1 at mitosis (32, 33), we mutated this amino acid to alanine (S71A) or aspartic acid (S71D) to mimic its unphosphorylated or phosphorylated state to determine if this site is important for the binding of LBR with importin β. Then we transiently expressed these two mutants in HeLa cells. The cells expressing GFP-xLBR1–210-S71D and GFP-xLBR1–210-S71D were synchronized, lysed, and subjected to pulldown assays as aforementioned. The result showed that unphosphorylation-mimicking mutant GFP-xLBR1–210-S71D could not bind with importin β, whereas the phosphorylation-mimicking mutant GFP-xLBR1–210-S71D bound importin β like the wild-type xLBR1–210 (control) (Fig. 2, D and E). Taken together, these results demonstrate that phosphorylation of xLBR at serine 71 regulates the interaction between LBR and importin β in mitosis.

We then asked whether disrupting the interaction between LBR and importin β interferes the NE assembly. We transiently expressed the full-length mutants GFP-LBR1–621-S71A, GFP-LBR1–621-S71D, and the wild-type GFP-LBR1–621 in HeLa cells followed by fluorescence microscopy. The result showed that, like the wild-type, both GFP-LBR1–621-S71D and GFP-LBR1–621-S71D caused no significant defects to the cells at interphase (data not shown). However, once the cell entered mitosis, the unphosphorylation-mimicking mutant GFP-LBR1–621-S71D showed a perturbed distribution and often aggregated into membrane stacks in the cytoplasm. At the end of mitosis (telophase/G1), this mutant protein did not become associated with the NE but remained in the membrane stacks (Fig. 2F). In contrast, the phosphorylation-mimicking mutant GFP-LBR1–621-S71D showed a similar NE distribution to the wild type at the end of mitosis (telophase/G1), except that the GFP-LBR1–621-S71D-expressing metaphase cells showed a likely continuous membrane around the aligned metaphase chromosomes (Fig. 2F). To explore and confirm whether serine 71 of LBR is phosphorylated during mitosis in HeLa cells, we performed phosphorylation reactions in vitro using his-hLBR1–210 and Cdc2-cyclin B. The sample was separated by SDS-PAGE, and the targeted gel band was cut, destained, and trypsin-digested. Then we analyzed the tryptic peptide mixtures using high resolution LTQ Orbitrap mass spectrometry and found that serine 71 was indeed phosphorylated (Fig. 2G). Together, these results indicate that the interaction of LBR with importin β regulated by phosphorylation at LBR Ser-71 is required for the NE membrane precursor vesicle targeting to the chromatin and the NE assembly. Deletion of the N-terminal Spanning Region Causes LBR Lateral Mobile and the Cell Death in Early G1, Due to the Failure of the NE Assembly—Knowing that the N-terminal spanning region of LBR is critical for its interaction with importin β and the NE assembly, we set out to examine the effects to the cells when deleting this N-terminal spanning region. We transfected the cells with the truncate GFP-xLBR1–621 and the full-length GFP-xLBR1–621 as control. We observed that, similar to the full-length GFP-xLBR1–621, the truncate GFP-xLBR1–621 caused no visible defects to the interphase cells at current cell cycle, no matter at low or high level expression (Fig. 3A). We immunostained the cells with a human LBR-specific antibody (Fig. 3B) and found that the endogenous LBR (in red) was interwoven with the truncate exogenous xLBR211–621 or the full-length foreign xLBR211–621 in the nuclear membrane (Fig. 3A, arrows). It is noteworthy that, similar to the full-length GFP-
xLBR\textsuperscript{1–621}, the truncate GFP-xLBR\textsuperscript{211–621} also induced the membrane stack formation in the cytoplasm and interweaved with the endogenous human LBR (red in Fig. 3A, arrowheads).

It has been reported that one role of the N-terminal region of LBR is to anchor this protein and the NE to the chromatin (39, 48, 49). In this work, we set out to determine whether the protein is mobile when the function of N-terminal region of LBR is disturbed. We carried out a FRAP experiment using the cells with low-to-middle level expression of the full-length GFP-xLBR\textsuperscript{1–210} as a control and the truncate GFP-xLBR\textsuperscript{211–621} (Fig.
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3, C and D). As expected, the fluorescence in the bleached NE regions of the full-length GFP-xLBR1–621-expressing cells could not recover within >300 s after the photo-bleaching, indicating that the full-length GFP-xLBR in the NE was totally immobilized as previously reported (48). In contrast, the majority of the truncate GFP-xLBR211–621 in the NE was mobile, as indicated by the fast recovery of the fluorescence of the photo-bleached region (Fig. 3D). This result suggests that the truncate GFP-xLBR211–621 is laterally mobile and that it moves rapidly from ER to the NE, although this truncate protein has no “root” to anchor itself to the chromatin and/or the nuclear lamina.

Because the truncate GFP-xLBR211–621 was mobile within the NE and showed no visible harm to the cell in current cell cycle, we wished to investigate if it is harmful to the cell in the next cell cycle. We transiently expressed the truncate GFP-xLBR211–621, full-length xLBR1–621 in HeLa cells and followed the behavior of the dividing cells. We found that, although GFP-xLBR211–621 is not significantly toxic to the cells in current cell cycle as aforementioned (Fig. 4A), the daughter cells overexpressing the truncate GFP-xLBR211–621 could not spread well after division (Fig. 4B), compared with the daughter cells overexpressing the full-length xLBR (data not shown). Furthermore, the truncate GFP-xLBR211–621 protein seemed to form vesicle-like or aggregate structures (Fig. 4B). By DAPI staining to visualize DNA, we found that the chromatin in these daughter cells could not decondense well, the NE around the chromatin could not be well assembled and the cells were likely dying (Fig. 4C). We repeated these experiments three times and found that the likely dying cells stood for ~70% of the paired G1 cells overexpressing the truncate GFP-xLBR211–621, whereas only 3% were dying among the paired cells expressing full-length xLBR1–621 (Fig. 4D). By time-lapse microscopy, we examined the behavior of the paired daughter cells during division (Fig. 4, E–G). We observed that the truncate GFP-xLBR211–621 and GFP-xLBR1–621ΔΔ69–73 did not take part in the NE assembly around the daughter nuclei, and the nuclei could not be well assembled, compared with those in the cells overexpressing full-length GFP-xLBR.

Furthermore, the daughter cells overexpressing GFP-xLBR211–621 and GFP-xLBR1–621ΔΔ69–73 could not spread well and finally showed apoptotic characteristics such as bubbling, chromatin condensing, and apoptotic body-like structure formation (Fig. 4, E and G).

To confirm if the dying paired G1 cells were apoptotic, we immunostained these cells with an antibody against the cleaved caspase 3, a marker for apoptotic cells (Fig. 5, A and B). We observed that the cleaved caspase 3 staining was totally negative in the mother cells (in the current cell cycle) and clearly positive in the paired cells (G1 phase of the next cell cycle). These results indicate that N terminus-deleted LBR and GFP-xLBR1–621ΔΔ69–73 cannot target the NE precursor membrane vesicles to the chromatin to form the NE, and the daughter cells without a functional NE would eventually die in G1 phase through apoptosis. Then we performed LBR RNAi and rescue experiment in HeLa cells to see if the function of LBR is critical for the NE assembly. At 72 h after siRNA transfection, the LBR protein level was decreased substantially detected by immuno-

FIGURE 3. GFP-xLBR211–621 could locate in the NE and induce endogenous LBR-containing membrane overproduction and stack formation in cytoplasm and these truncated proteins are laterally mobile.

A, HeLa cells overexpressing full-length GFP-xLBR1–621 or the N-terminal spanning region-deleted truncate GFP-xLBR211–621 were fixed and immunostained with hLBR-specific antibody, and counterstained with DAPI for DNA. Note that both the full-length and the truncated LBR localized on the NE (arrows) and induced membrane overproduction and stack formation containing endogenous LBR in the cytoplasm (arrowheads). B, characterization of anti-human hLBR antibody used in A. This antibody specifically recognizes endogenous hLBR but not the exogenous GFP-xLBR. C, FRAP assay for the full-length GFP-xLBR1–621 on the NE. Note that the fluorescence in the photo-bleached regions could not recover within as long as 300 s, indicating that the full-length xLBR1–621 was static on the NE. D, FRAP assay for the GFP-xLBR211–621 on the NE. Note that the fluorescence in the photo-bleached regions recovered rapidly, indicating that GFP-xLBR211–621 was mobile on the NE. Bars, 10 μm.

xLBR211–621 is not significantly toxic to the cells in current cell cycle as aforementioned (Fig. 4A), the daughter cells overexpressing the truncate GFP-xLBR211–621 could not spread well after division (Fig. 4B), compared with the daughter cells overexpressing the full-length xLBR (data not shown). Furthermore, the truncate GFP-xLBR211–621 protein seemed to form vesicle-like or aggregate structures (Fig. 4B). By DAPI staining to visualize DNA, we found that the chromatin in these daughter cells could not decondense well, the NE around the chromatin could not be well assembled and the cells were likely dying (Fig. 4C). We repeated these experiments three times and found that the likely dying cells stood for ~70% of the paired G1 cells overexpressing the truncate GFP-xLBR211–621, whereas only 3% were dying among the paired cells expressing full-length xLBR1–621 (Fig. 4D). By time-lapse microscopy, we examined the behavior of the paired daughter cells during division (Fig. 4, E–G). We observed that the truncate GFP-xLBR211–621 and GFP-xLBR1–621ΔΔ69–73 did not take part in the NE assembly around the daughter nuclei, and the nuclei could not be well assembled, compared with those in the cells overexpressing full-length GFP-xLBR.

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blotting (Fig. 5C). We also co-transfected the cells with siRNA and RFP-H2B as a transfection reporter for microscopy. After 72 h, the cells were stained with an antibody against cleaved caspase 3. We found that LBR RNAi caused apoptosis of the daughter cells (Fig. 5D) and that the cells could be rescued by expressing RNAi-resistant xLBR (Fig. 5E). Taken together, these results demonstrate that the function of LBR is crucial for the NE assembly.

The First Transmembrane Domain of LBR Induces the NE Membrane Production and Targeting to the Chromatin—It has been previously shown that overexpression of exogenous full-length or N-terminal spanning region-deleted LBR in HeLa cells results in membrane overproduction and stack formation in the cytoplasm (15). To reveal how the membrane overproduction and stack formation were induced, we constructed GFP-fused full-length xLBR1–621 and a number of truncated proteins: GFP-xLBR1–210 (the N-terminal spanning region), GFP-xLBR1–260 (the N-terminal spanning region plus the first transmembrane domain), GFP-xLBR211–621 (xLBR without the N-terminal spanning region), GFP-xLBR261–621 (xLBR without the N-terminal spanning region and the first transmembrane domain), GFP-xLBR2211–289 (xLBR without the first and the second transmembrane domains) (Fig. 6A). We transiently expressed these proteins in HeLa cells and observed their distributions in the cells. The results showed that overexpressing full-length GFP-xLBR1–621 and GFP-xLBR211–621 efficiently induced membrane over-generation and stack formation and that the N-terminal spanning region was situated in the nucleus when expressed (Fig. 6B). Furthermore, we observed that the truncate xLBR1–260, which only contains the N-terminal spanning region and the first transmembrane domain, not only localized to the

![Image](image_url)
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Overexpression of GFP-xLBR1–621 and GFP-xLBR211–621 results in the daughter cell death at early G1 phase. A, HeLa cell transiently expressed GFP-xLBR211–621 in the presence of 2.5 mM thymidine in the medium, which was used to arrest the cells at interphase, were fixed and viewed directly. Note that the GFP-xLBR211–621-expressing cells were alive in interphase in current cell cycle. Bar, 10 μm. B, four paired representative likely dying early G1 cells were fixed and viewed directly. Consistently, we also observed that both the truncate proteins GFP-xLBR211–260 (both the first and the second transmembrane domain deletion truncate mutant) and GFP-xLBR211–289 (both the first and the second transmembrane domain deletion truncate mutant) localized throughout the cell but not the NE and did not induce membrane stack formation (Fig. 6B). These results not only confirm that the first transmembrane domain of LBR is essential for the NE localization of this protein as reported previously (39, 50), but also demonstrate that the transmembrane domain induces the membrane overproduction and the stack formation.

FIGURE 5. Dysfunction or knockdown of LBR causes the cell death in early G1 phase via apoptosis. A, the upper panel shows a mother cell, and the lower panels show three paired daughter cells. Note that overexpression of GFP-xLBR211–621 does not significantly harm the mother cell but causes the daughter cell death during the next G1 phase through apoptosis, judging by positive staining of the cleaved caspase 3, an apoptosis marker. Bars, 10 μm. B, the upper panel shows a mother cell, and the lower panels show three paired daughter cells. Note that Overexpression of GFP-xLBR1–210 does not significantly harm the mother cell but causes the daughter cell death during next G1 phase through apoptosis. Bars, 10 μm. C, HeLa cells transfected with control or siRNA were collected at 72 h after transfection and analyzed by Western blot with antibodies against human LBR and α-tubulin serving as a loading control. D, HeLa cells co-transfected with control, siRNA, and RFP-H2B (1:20) were immunostained with anti-cleaved caspase 3 antibody (green) at 72 h after transfection. The cells deleted endogenous LBR died in early G1 phase, judging by cleaved caspase 3-positive staining. E, HeLa cells co-transfected with siRNA, GFP-xLBR1–210-S71D, and RFP-H2B, and siRNA-resistant GFP-xLBR. The cells co-transfected with siRNA, GFP-xLBR1–210-S71D, and RFP-H2B showed a normal cell division, indicating that expression of the siRNA-resistant xLBR rescued the cell death caused by hLBR knockdown.
it may not block the targeting of LBR-containing membrane vesicles to the chromatin for the NE assembly by importin β. Because S71D could bind with importin β (see Fig. 2E), it may block the targeting of LBR-containing membrane vesicles to the chromatin by importin β for the NE assembly. Therefore, these results demonstrate that, even in the in vitro NE assembly system based on *Xenopus* egg extracts, phosphorylation of Ser-71 of LBR also regulates the binding of LBR and importin β for the LBR-bearing membrane targeting to the chromatin and the NE assembly.

**DISCUSSION**

Importin β is an evolutionarily conserved protein that serves as a main nucleocytoplasmic transport receptor to regulate the nuclear import together with importin α regulated by Ran (51–56). Importin β can also mediate the nuclear import of some proteins independently of importin α (57–63). It is also found that, in early mitosis, importin β plays an inhibitory role for mitotic spindle assembly by interacting with and suppressing the spindle assembly factors (64), and this inhibitory role can be eliminated by Ran-GTP, which interacts with importin β and releases the spindle assembly factors (6). At the end of mitosis, importin β takes part in the NE assembly (9, 12, 15, 65). Importin β was first reported to take a role in the NE assembly in *Xenopus* egg extracts in an appropriate dosage-dependent manner, and if its concentration was increased in the extract by adding excess importin β protein, it inhibits the NE assembly (9, 12, 13). In our previous report, we found that one role of importin β in the NE assembly is to target the NE membrane precursor vesicles to the chromatin through interaction with the membrane-born LBR (15). We found that depletion of LBR causes the cell death via apoptosis at early G1 phase due to the NE assembly failure. We have pinpointed the binding regions, which are aa 69–90 in LBR and aa 45–462 in importin β. We also demonstrate that the interaction of both proteins is regulated by phosphorylation of LBR at Ser-71 and that the interaction plays a crucial role in mediating the LBR-containing membrane vesicle targeting to the chromatin for the NE assembly.

LBR has been implicated in a number of important functions. It plays roles in sustaining the NE architecture, in NE assembly, in the lobulated (segmented) nuclear shape formation in the development of the neutrophiles, and in the regulation of proliferative and functional responses for innate immunity (66–69). It is also involved in some diseases, such as Greenberg skeletal dysplasia and Pelger-Huet anomaly (38, 70–72). However, the detailed analyses of LBR have been performed using its soluble and functional N-terminal spanning region, which provides essential chromatin docking sites at the NE (22, 24, 30, 73). During NE assembly, the N-terminal spanning region of LBR targets its associated membrane vesicles to the chromatin by interacting with importin β (15). In this work, we have identified the interacting sites in both importin β and LBR N-terminal spanning region. We observed that, without the N-terminal spanning region, LBR is mobile compared with the stock-still full-length LBR at the NE. Even so, the N-terminal spanning region-deleted LBR is not immediately lethal to the cell in the current cell cycle, and the cell could go to mitosis. However, when the cell divides, its daughter cells could not

spanning region His-xLBR<sub>1–210</sub> and its mutants were added, we found that the wild-type and the mutant S71D, but not S71A, blocked the NE assembly around the sperm chromatin (Fig. 7, B–D). To understand these results, we supposed that, because S71A mutant did not bind with importin β (see Fig. 2, D and E),
organize their complete and functional NE due to the LBR-bearing NE membrane precursor vesicles not being targeted onto the chromatin, and their chromatin could not be fully decondensed. Finally, these daughter cells will die in early G1 phase by apoptosis. This result demonstrates that LBR does play a role in the NE assembly. Besides, LBR may play a role in chromosome decondensation and nuclear structure organization through anchoring the chromatin to the NE and organizing local domains through binding some factors like lamin B, HP1, and chromatin/DNA for some special nuclear functions (25, 28, 30). To perform these functions, LBR may be regulated through some modifications. During interphase, the RS region of LBR is mainly phosphorylated by NE-bound kinase-RS kinases; whereas in mitosis, the RS region is phosphorylated by NE-bound kinase-RS domain of LBR is responsible for the NE generation. We propose that, during the NE assembly at the end of mitosis, the complex of importin β/LBR-bearing membrane vesicle complex approaches the surface of the chromatin, importin β preferentially binds with Ran-GTP on the surface of the chromatin generated by RCC1 and releases the LBR-bearing membrane vesicle. Simultaneously, PP1 dephosphorylates LBR at Ser-71 and prevents it from rebinding with importin β but to the chromatin to form the NE.

Once the NE is assembled around the daughter nuclei, it grows to meet the requirement of the chromatin decondensation to a regular size and the function of the interphase nucleus. It has been shown that overexpression of LBR in yeast cells induced membrane overproduction and stack formation (40). We have previously reported that, in mammalian cells, overexpression of LBR resulted in overproduction of the NE, which either invaginated into the nucleus or budded off from the NE into the cytoplasm in an expression quantity-dependent manner (15). In this work, we found that the first transmembrane domain is an essential part for the NE growth and the membrane stack formation. With the N-terminal spanning region, the first transmembrane domain could induce the membrane stack formation when it is overexpressed. If the first transmembrane domain is deleted, the deletion mutant will not be able to localize to the NE, and its overexpression will not cause any membrane overproduction and membrane stack formation. Therefore, we propose that the first transmembrane domain of LBR is responsible for the NE generation and localization to the NE.

In summary, we found that the interaction between importin β and the N-terminal spanning region of LBR occurs between aa 45–462 of importin β and aa 69–90 of LBR and that the interaction is regulated by the phosphorylation at the amino acid serine 71 of LBR. Disruption of this interaction by deletion of the N-terminal spanning region or aa 69–73 aa of LBR inhibits the NE assembly. We also found that the first transmembrane domain of LBR is responsible for the NE generation. We propose that, during the NE assembly at the end of mitosis, LBR-containing NE precursor membrane vesicles are targeted to the chromatin by the N-terminal spanning region of LBR through binding with importin β in a LBR Ser-71 phosphorylation-dependent manner. Once it approaches the surface of the chromatin at the end of mitosis, the complex of importin β/LBR disassociates through the preferential binding of importin β with Ran-GTP and the dephosphorylation of the released LBR
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by PP1 to prevent LBR from rebinding to importin β. The unphosphorylated LBR with the membrane vesicles binds to the chromatin through its N-terminal spanning region, and the vesicles fuse to form the NE. Without a functional N-terminal spanning region, the NE assembly by LBR-containing NE precursor membrane vesicles and importin β would be inhibited, and the daughter cells would die in the next G1 phase through apoptosis. LBR may also perform a role for the nuclear growth and the NE expansion through its first transmembrane domain in interphase.

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