Lamin B receptor plays a role in stimulating nuclear envelope production and targeting membrane vesicles to chromatin during nuclear envelope assembly through direct interaction with importin β

Yan Ma, Shang Cai, Quanlong Lv, Qing Jiang*, Quan Zhang, Sodmergen, Zhonghe Zhai and Chuanmao Zhang*

The Key Laboratory of Cell Proliferation and Differentiation of Ministry of Education, The National Key Laboratory of Bio-membrane and Membrane Biotechnology, the College of Life Sciences, Peking University, Beijing 100871, China

*Authors for correspondence (e-mail: qingjiang@pku.edu.cn; zhangcm@pku.edu.cn)

Accepted 21 November 2006
Journal of Cell Science 120, 520-530 Published by The Company of Biologists 2007
doi:10.1242/jcs.03355

Summary

Lamin B receptor (LBR), a chromatin and lamin binding protein in the inner nuclear membrane, has been proposed to play a vital role in nuclear envelope (NE) assembly. But the specific role for LBR in NE assembly remains unknown. In the present study, we show that overexpression of LBR causes membrane overproduction, inducing NE invagination and membrane stack formation, and that these processes require the transmembrane domain of LBR. Biochemical analysis shows that the N-terminal domain of LBR directly interacts with importin β in a Ran sensitive and importin α independent manner. Using an in vitro NE assembly assay, we also demonstrate that blocking full length LBR binding sites on importin β, by the addition of the LBR N-terminal domain inhibits the recruitment of LBR-containing vesicles to importin β- or Ran-coated beads to form NE structure. Our results suggest that LBR is recruited to chromatin through direct interaction with importin β to contribute to the fusion of membrane vesicles and formation of the NE.

Key words: Nuclear envelope assembly, Lamin B receptor, Importin β, Ran GTPase

Introduction

The nuclear envelope (NE) is highly dynamic during the cell cycle. In metazoan cells, the NE breaks down at the onset of prometaphase and the membrane fragments containing nucleoporins or integral membrane proteins disperse into the cytoplasm. At the end of mitosis, the NE reassembles and grows on the surface of the decondensing chromatin, forming two new nuclei (Gant and Wilson, 1997). The mechanism for NE formation and growth has been studied for over half a century, and recently it was shown that Ran, a small Ras-like nuclear GTPase, regulates NE assembly. Specifically, beads coated with RanGTP are able to assemble functional NEs in the absence of chromatin in a cell-free system and require the hydrolysis of the GTP molecule on Ran (Zhang and Clarke, 2000; Hetzer et al., 2000). Subsequent studies showed that importin β, a Ran-binding protein that plays a role in nucleocytoplasmic transport and spindle assembly, is also involved in Ran-regulated NE assembly. Importin β-depleted *Xenopus* egg extract fails to support NE assembly around beads coated with Ran, and the failure could be rescued by adding back bacterially expressed importin β in a concentration-dependent manner (Zhang et al., 2002b). In *Caenorhabditis elegans*, embryos depleted of importin β by RNAi show a strong defect in membrane recruitment to the reforming NE (Askjaer et al., 2002). Most importantly, the importin β-coated beads themselves were able to directly induce NE assembly in *Xenopus* egg extracts (Zhang et al., 2002b). These results demonstrate that Ran and importin β play important roles in NE assembly.

Although Ran and its binding proteins are crucial for NE assembly, the mechanism for NE precursor vesicle recruitment to the chromatin is poorly understood (Zhang and Clarke, 2000; Zhang et al., 2002a; Zhang et al., 2002b), and the downstream effectors are at best vaguely known. For example, importin β interacts with the FXFG domain of nucleoporins (Shah et al., 1998; Bayliss et al., 2000) and Ran and its binding proteins regulate the nuclear pore complex formation by targeting the nucleoporins to chromatin (Zhang et al., 2002a; Walther et al., 2003). In addition, there are a number of integral membrane proteins localized at the inner NE that are responsible for targeting the precursor vesicles to chromatin during NE assembly. It is likely that some of these inner NE proteins are the downstream effectors of Ran and its binding proteins. Among those integral membrane proteins, the lamin B receptor (LBR) appears to be a central player in targeting nuclear membranes to chromatin (Gant and Wilson, 1997) and therefore is a good candidate as a target of importin β.

LBR is an evolutionally conserved and developmentally essential inner nuclear membrane protein, ubiquitous in vertebrates, *Drosophila* and yeast (Worman et al., 1988; Wagner et al., 2004). LBR consists of a hydrophilic N-terminal domain, a short hydrophobic C-terminal domain and eight predicted transmembrane segments. Both the C-terminal and the N-terminal domains project into the nucleoplasm (Worman

et al., 1988; Worman et al., 1990). The LBR transmembrane segments show a significant sequence similarity to vertebrate, yeast and plant sterol reductases, which form a multigene family (Holmer, 1998; Silve, 1998; Georgatos, 2001). The N-terminal domain of LBR binds to B-type lamins, chromosomes/chromatin and DNA, and interacts with human heterochromatin protein HP1 (Dreger et al., 2002; Duband-Goulet and Courvalin, 2000; Makatsori et al., 2004; Meier and Georgatos, 1994; Pyrpasopoulou et al., 1996; Kawahire et al., 1997; Gajewski and Krohne, 1999; Simos and Georgatos, 1992, Ye and Worman, 1994; Ye and Worman, 1996; Ye et al., 1997). The binding between LBR and chromatin is cell cycle-dependent and regulated by phosphorylation through multiple kinases (Takano et al., 2002). The N-terminal domain of LBR contains multiple serine-arginine motifs that are phosphorylated by the SRPK1 and cdc2 kinases (Nikolakaki et al., 1996; Nikolakaki et al., 1997; Takano et al., 2002). These features make LBR an interesting player in nuclear assembly. Live imaging of GFP-LBR-expressing cells show that LBR disperses into the cytoplasm at early mitosis in metazoans, and is recruited to the decondensing chromatin at the early stages of nuclear reformation (Ellenberg et al., 1997). In addition, LBR and a LBR-like integral membrane protein of sea urchins target membranes to the chromatin surface (Collas et al., 1996; Drummond et al., 1999; Ellenberg et al., 1997; Meier and Georgatos, 1994; Pyrpasopoulou et al., 1996). Of medical significance, mutation of the LBR gene causes developmental abnormalities, reduced survival of homozygous embryos and serious hereditary diseases (Shultz et al., 2003; Waterham et al., 2003).

In an attempt to gain a further insight into the role of LBR in NE assembly, we investigated the nuclear membrane dynamics in LBR-overexpressing cells. We also identified a novel interaction between LBR and importin β, and reveal that this interaction is important for nuclear assembly. We show that LBR, an inner integral nuclear membrane protein, is regulated by Ran and recruits membrane vesicles to chromatin during the assembly of the NE.

Results

Overexpression of GFP-xLBR causes NE over-production

NE assembly is one of the key steps in generating daughter nuclei during the cell division in eukaryotic cells. LBR has been thought to have a role in the NE assembly through sorting and targeting NE membranes to chromatin (Smith and Blobel, 1993; Collas et al., 1996; Pyrpasopoulou et al., 1996). By fusing with GFP, the behavior of truncated and full length LBR have been observed during NE dynamics (Ellenberg et al., 1997; Haraguchi et al., 2000; Irons et al., 2003). Despite these and other advances, important questions concerning the mechanism of NE assembly and dynamics still remain largely unanswered.

To study the mechanism of NE assembly, we cloned the *Xenopus* LBR gene and transiently expressed GFP-xLBR in human HeLa cells (Fig. 1A,B). In HeLa cells expressing relatively low levels of GFP-xLBR, the GFP-xLBR fusion proteins have been found to be mainly located on the NE with a small portion in the cytoplasm, similar to that reported earlier for mammalian LBR (Ellenberg et al., 1997; Haraguchi et al., 2000). We followed the dynamics of this fusion protein during the cell cycle and found that the *Xenopus* protein, like human LBR, did not disturb the cell cycle at low expression levels (Fig. 1A). GFP-xLBR dispersed into the cytoplasm when the cell went into mitosis and started to rebind to the surface of the daughter chromosomes starting in late anaphase, consistent with that observed for human LBR (Ellenberg et al., 1997). This data suggests that the behavior of xLBR in HeLa cells during the cell cycle is similar to that of human LBR.

We then analyzed the relationship between the expression level of GFP-xLBR and membrane dynamics. In cells expressing low levels of GFP-xLBR, the NE had no significant

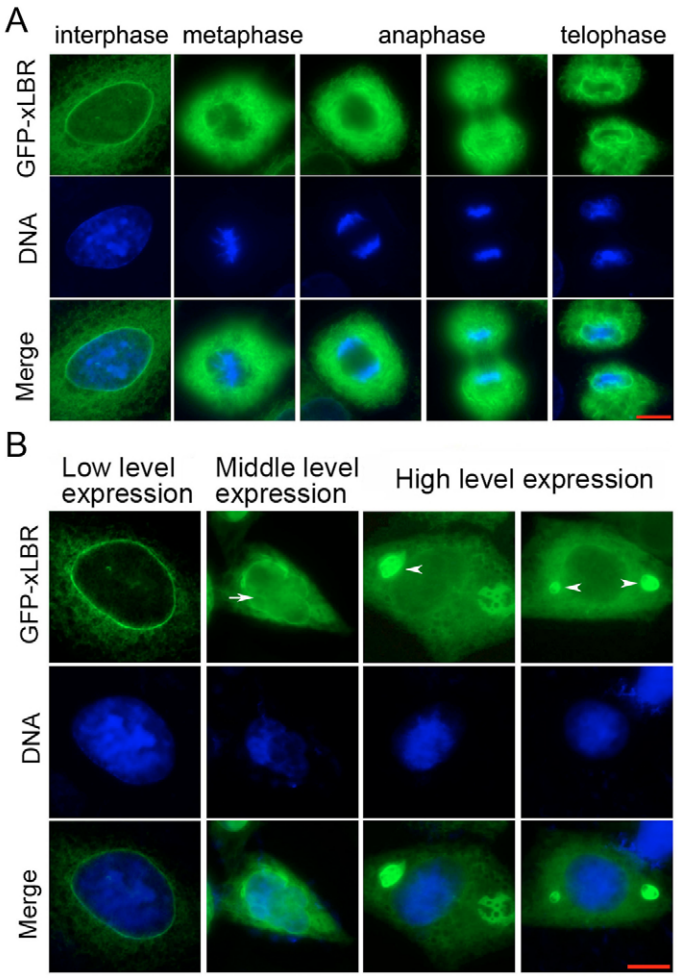

Fig. 1. GFP-xLBR overexpression leads to nuclear membrane overproduction. (A) Location of GFP-xLBR expression during the cell cycle. HeLa cells were transfected with GFP-xLBR expression vector and visualized under a fluorescence microscope to judge the expression levels of the fusion protein. HeLa cells expressing low levels of full-length GFP-xLBR of various stages of cell cycle were fixed and visualized for GFP or stained with DAPI for DNA. Note that the fusion protein was located to the NE and ER in interphase and had normal cell cycle distribution dynamics. (B) High level expression of GFP-xLBR caused nuclear membrane overproduction. Note that with increasing expression of GFP-xLBR, the excess NE either folded into the nucleoplasm (arrows) and/or formed vesicular aggregates (arrowheads) in the cytoplasm. DNA was stained blue with DAPI. Bars, 10 μm.

changes, compared with that of untransfected cells. Nevertheless, when the protein was transiently expressed to an intermediate level, it caused an over-production and folding of the NE into the nucleoplasm, and when expressed to a high level, large GFP fluorescent vesicles aggregates formed outside the nucleus (Fig. 1B). This phenomenon was also observed by transfecting the cells with HA-tagged LBR followed by immunofluorescence staining with an anti-HA-tag antibody (data not shown). This suggests that LBR could be saturated in the NE. If there was more LBR than the NE could house, extra LBR could force the NE to over-generate and protrude into the nucleoplasm and/or the cytoplasm, arguing that LBR may be important for nuclear membrane growth.

The vesicular aggregates caused by LBR overexpression are composed of membrane stacks from the NE

To determine if the vesicle aggregates came from the NE, we analyzed the aggregate formation process by time-lapse microscopy. We first observed that a pocket-like structure projected from the NE and that the edges of the stack were continuous with the NE (Fig. 2A). Along with its growth, more GFP-xLBR accumulated in the pocket. The neck of the pocket gradually narrowed, and finally, the stack dropped from the NE. Interestingly, in the same cell, we could see two small vesicular aggregates fuse into one, indicating that the large aggregates might come from the fusion of smaller ones. These results suggest that the vesicle aggregates came directly from the NE.

To determine the structures of the NE and the vesicles in the presence of GFP-xLBR overexpression, we performed correlative light and transmission electron microscopy (TEM) at the single cell level (Fig. 2B). We found no significant differences at the ultra-structure level between the NE with GFP-xLBR and the NE without GFP-xLBR expression. By contrast, the GFP-xLBR vesicle 'membrane' consisted of bi-layered onion-like membrane stacks (Fig. 2B). The paired membranes looked like the NE, but there were no obvious nuclear pore complexes. To confirm that the membrane stacks did not contain nucleoporins or associated lamin B, we stained the cells with anti-nucleoporins mAb414 and anti-lamin B antibodies, and found none of these proteins in the stacks (Fig. 2C,D). We also stained the cells with antibodies against a Golgi marker and an ER marker in order to investigate if there are similarities between the membrane stacks and Golgi/ER. The immunofluorescence images indicated that the membrane stacks contained the ER marker but lacked the Golgi marker (Fig. 2E,F).

The transmembrane segment of LBR is responsible for membrane overproduction, and the N terminus of LBR is required for NE invagination

We next asked how overexpression of xLBR resulted in the membrane overproduction. To answer this question, we generated a number of vectors containing distinct lengths of the LBR gene and transiently expressed the truncated forms of this protein in HeLa and XTC cells (Fig. 3A,B). The transfection results showed that full-length GFP-xLBR, GFP-xLBR$^{1\text{-}210}$, GFP-xLBR$^{211\text{-}621}$ had the same distribution patterns as described previously for similar truncations of chicken LBR

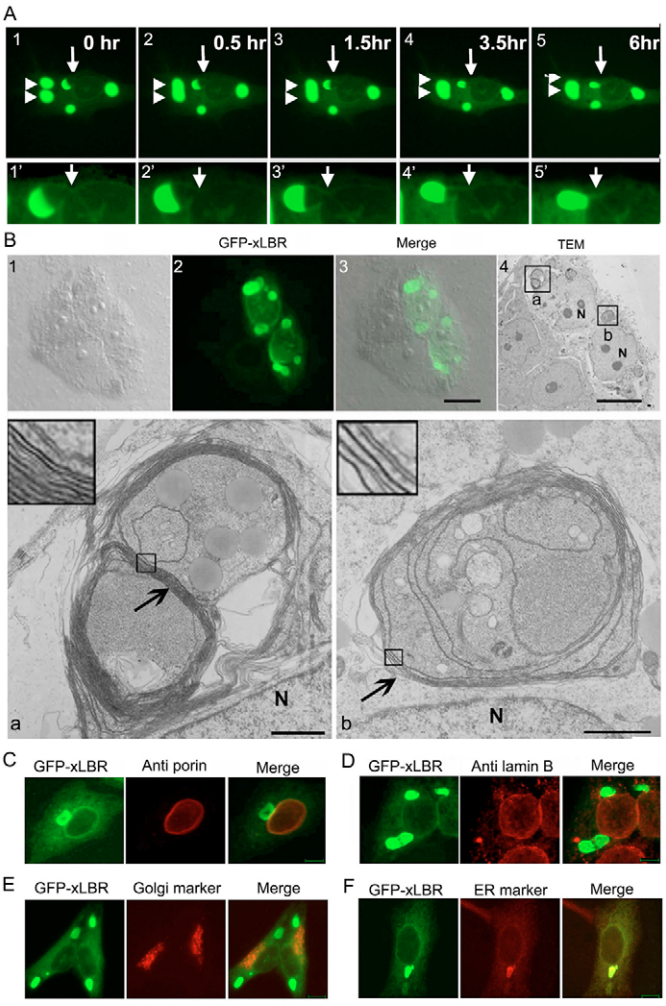

Fig. 2. Perinuclear aggregates of GFP-xLBR bud off from the nuclear membrane and form vesicles with membrane stacks that do not contain lamin B or nucleoporins. (A) HeLa cells overexpressing GFP-xLBR were viewed by time-lapse microscopy. At the beginning, the vesicle was very small and was connected through its edges with the nuclear membrane (NE, arrows). Within several hours, the vesicle gradually and progressively pinched off from nuclear membrane. Also, the small vesicle aggregates could fuse to form a large one (arrowheads). The area indicated by the arrow in the top panels was enlarged and shown in the bottom panels. Bar, 10 μm. (B) HeLa cells overexpressing GFP-xLBR were visualized by fluorescence microscopy and then processed for TEM. The nuclear membrane in the transfected cells appeared normal (B1-B4; the two transfected and two nontransfected cells look similar). TEM examination of aggregates showed that vesicles had numerous bi-layered stacks of NE-like membranes (indicated by arrows). In B4 the boxed areas a and b are shown at higher magnification in Ba and Bb. The insets in a and b are higher magnification of the boxed areas. N, nucleus. Bars, 10 μm in the upper panels and 1 μm in the lower panels. (C,D) HeLa cells overexpressing GFP-xLBR were fixed and stained with anti-nucleoporin monoclonal antibody mAb414 (C) or anti-lamin B (D). Neither the nuclear pore complex component nor Lamin B was observed on the membrane stacks.
(E,F) Immunofluorescence of HeLa cells overexpressing GFP-xLBR using the antibody against a Golgi marker (E) or the ER marker (F). Bar, 10 μm.

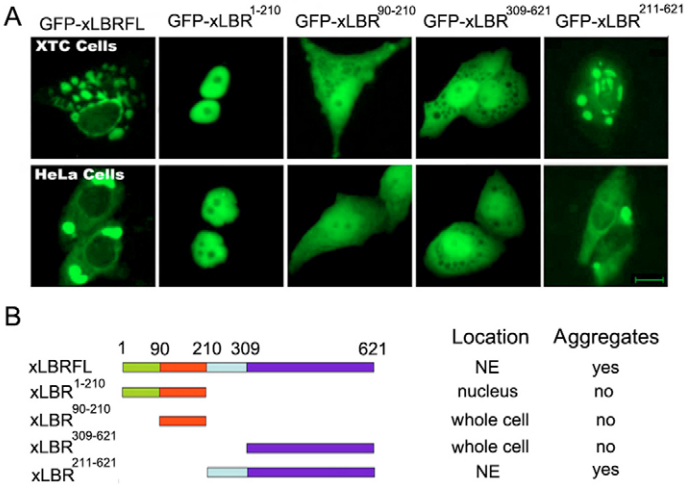

Fig. 3. xLBR domain requirement for NE localization and aggregation production in both HeLa and XTC cells. (A) GFP-fused LBR or its deletion mutants were overexpressed in HeLa or XTC cells. The cells were examined under a fluorescence microscope. Note that GFP-xLBR$^{1-210}$ was concentrated in nucleoplasm, whereas the GFP-LBR$^{90-210}$ was distributed throughout the whole cell, indicating that a nuclear localization signal is present within amino acids 1-90. GFP-LBR$^{211-621}$ was located on the nuclear rim and forms aggregates although less than those of full length xLBR, whereas GFP-LBR$^{309-621}$ was located in the whole cell body, suggesting that the first transmembrane segment within amino acids 211 to 309 is necessary for locating the protein to the NE and formation of the aggregates. Bar, 10 μm. (B) A schematic diagram of the domain structure of LBR.

(Soullam and Worman, 1993). Specifically, GFP-xLBR$^{1-210}$ was localized to the nucleus only and did not cause aggregate formation upon overexpression, whereas GFP-xLBR$^{211-621}$ was localized to the NE and caused aggregates upon overexpression, indistinguishable from full-length GFP-xLBR (Fig. 3). By contrast, GFP-xLBR$^{90-210}$ localized within the entire cell body (nucleus and cytoplasm), indicating that the fragment from residue 1 to residue 90 is responsible for its nuclear retention. Similar to GFP-xLBR$^{90-210}$, GFP-xLBR$^{309-621}$ was distributed within the entire cell body. Only full length GFP-xLBR and GFP-xLBR$^{211-621}$ induced the membrane stacks. Since GFP-xLBR$^{309-621}$ could not induce membrane stacks, we suggest that the membrane-targeting segment between amino acids 211 and 308 is required for the nuclear membrane production as well as for targeting the protein to the NE. The results indicated that the transmembrane segment of LBR may have novel activity that promotes NE overgrowth when LBR is overexpressed.

Interestingly, in GFP-xLBR$^{211-621}$-expressing cells, although we did see the membrane stack formation, we failed to observe NE invagination as found with full length GFP-xLBR. This suggests that the N terminus of LBR is required for NE invagination in LBR-overexpressing cells, possibly due to the known interaction of the N terminus with chromatin or chromatin-associated proteins (Pyrpasopoulou et al., 1996; Ye and Worman, 1994). We inferred that the transmembrane segment of LBR participates in the overproduction of membrane and that the N terminus of LBR facilitates the attachment of the overgrown membrane to the chromatin, leading to invaginations of the NE.

The N-terminal domain of lamin B receptor can bind directly to importin β

The ability of LBR to stimulate nuclear membrane growth and link the membrane to chromatin prompted us to study the pathway of LBR recruitment to chromatin at the end of mitosis, including the identification of interacting partner proteins. Importin β is known to play a vital role in vesicle recruitment during NE assembly (Zhang et al., 2002b). Because LBR disperses into the cytoplasm after the NE breaks down and participates in the NE reassembly at telophase, the possibility exists that the recruitment of vesicles containing LBR to the reforming NE is mediated by importin β. To test whether importin β and LBR bind each other, we firstly generated the antibody against hLBR$^{1-60}$, which can detect human LBR very well (data not shown). Then we carried out an immunofluorescence microscopic study and confirmed that part of importin β colocalized with LBR in interphase cells. During NE assembly, both importin β and LBR were recruited and colocalized on the chromatin (Fig. 4A). We further performed a pull-down experiment using mitotic HeLa extract. We loaded equal amounts of purified His-GFP or His-GFP-LBR$^{1-210}$ onto Sepharose beads and incubated the beads with the extract, followed by centrifugation to isolate the beads. Proteins on the beads were separated on a gel and analyzed by western blot with importin β antibody. The result showed that His-GFP-LBR$^{1-210}$ but not His-GFP specifically pulled down the importin β protein (Fig. 4B). Similarly when importin β beads were incubated with the purified GFP-LBR$^{1-210}$, the fluorescent LBR$^{1-210}$ could be clearly observed around the importin β but not the control GST beads, indicating that importin β and LBR interacted with each other (Fig. 4C). Finally we investigated the strength of the binding interaction between GFP-xLBR$^{1-210}$ and GST-importin β by washing the beads with different concentrations of NaCl. We discovered that GST-importin β bound to the N terminal domain of LBR in binding buffer containing 100 mM or 300 mM NaCl, but 500 mM NaCl abolished the interaction (Fig. 4D). These results demonstrated that the interaction between LBR and importin β is direct and can stand for at least 300 mM NaCl. This was also supported by the ability of the N terminus of xLBR to specifically pull down importin β from *Xenopus* egg extract as revealed by silver-stained gel (Fig. 4E) and western blotting using the anti-importin β antibody (Fig. 4F).

Amino acids 45-90 are crucial for the interaction between xLBR and importin β

To investigate which part of xLBR is responsible for the novel interaction, we constructed a series of N-terminally truncated xLBR proteins: His-xLBR$^{45-210}$, His-xLBR$^{53-210}$, His-xLBR$^{81-210}$ and His-xLBR$^{90-210}$ (Fig. 5A) and performed *in vitro* binding assays with GST-importin β to determine their interactions with importin β. We found that importin β bound to xLBR$^{45-210}$ as efficiently as xLBR$^{1-210}$ (Fig. 5B,C). By contrast, xLBR$^{53-210}$ and xLBR$^{81-210}$ had increasingly reduced affinity for importin β and xLBR$^{90-210}$ had none (Fig. 5B,C).

The above experiments indicated that sequences after amino acids 45 were important for the binding of xLBR to importin

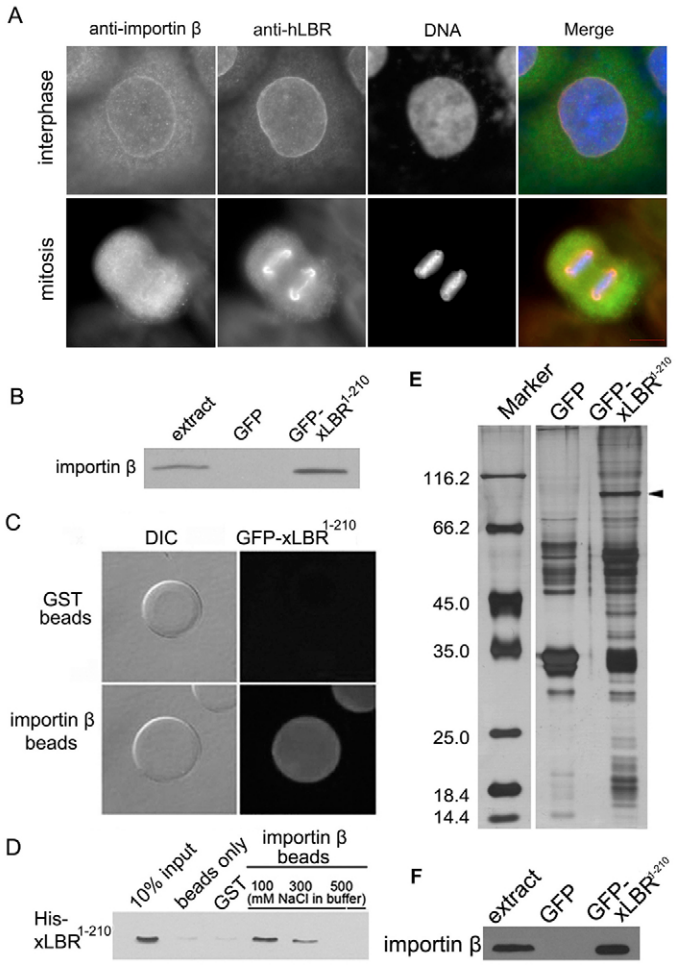

Fig. 4. LBR binds to importin β in vivo through the N-terminal 1-210 domain. (A) Interphase and mitotic HeLa cells were analyzed by indirect immunofluorescence microscopy using anti-importin β and LBR antibodies, and shows colocalization of these endogenous substances. (B) The N-terminal domain of xLBR binds to importin β in mitotic HeLa extract. CNBr-activated Sepharose 4B-coated with GFP-xLBR$^{1-210}$ was incubated with mitotic HeLa extract. The proteins bound to the beads were isolated and subjected to western blot analysis for the presence of importin β. (C) A fluorescence protein binding assay shows direct interaction of LBR with importin β. Purified GFP-xLBR$^{1-210}$ bound to importin β but not GFP beads. (D) The purified N-terminal domain of xLBR binds to importin β in the presence of 100 mM or and 300 mM NaCl but not in the presence of 500 mM NaCl. Proteins associated with the importin β beads were subjected to western blot with anti-His antibody. (E) In *Xenopus* egg extract, GFP-xLBR$^{1-210}$ but not GFP beads could specifically pull-down the endogenous importin β (arrowhead). Proteins associated with the beads were separated on a SDS gel followed by silver staining. The molecular mass markers are shown on the left. (F) Western blot analysis of the precipitated protein in E and egg extract for the presence of importin β.

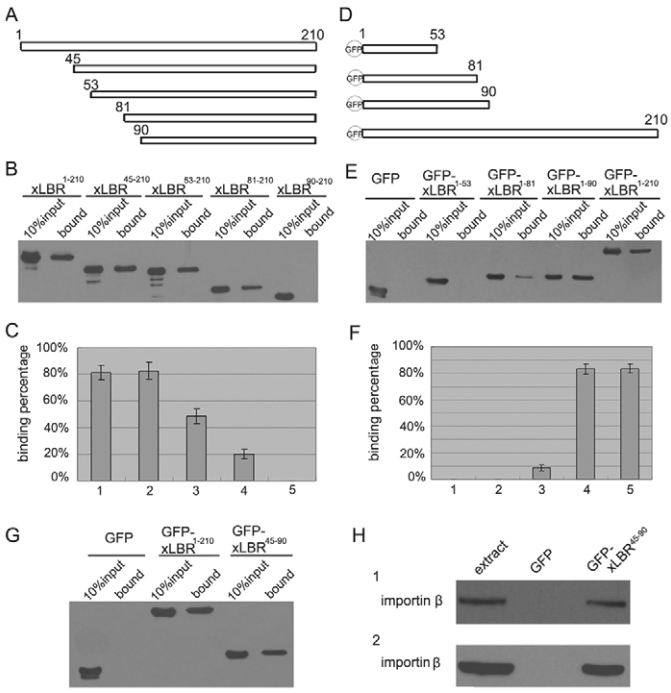

Fig. 5. LBR binds to importin β via amino acids 45 to 90. (A) Schematic diagram of N-terminal deletion constructs of xLBR$^{1-210}$. The cDNAs for the different truncated N-terminal domains of xLBR were subcloned into pET28a to express the devised polypeptides. (B) Western analysis of the in vitro binding assay using the truncated LBR fragments and GST-importin β. Note that deletion past aa 45 reduced the binding, and past aa 90 abolished binding. (C) Quantification of the in vitro binding assayed in B by densitometry. 10% of the input for each fragment was set to 100%. The data is shown as the mean percentage bound plus the standard deviation. (D) Schematic diagram of the C-terminal deletion mutants of xLBR$^{1-210}$. The cDNAs for the different domains of xLBR were subcloned into pET28a-GFP to express the polypeptides fused with GFP. (E) In vitro binding assay using the C-terminally truncated LBR proteins and importin β. Note that GFP-xLBR$^{1-53}$ did not bind to importin β and GFP-xLBR$^{1-81}$ had weak binding. (F) Quantification of the in vitro binding assayed in E, which was identical to the method used in C. (G) Analysis of the binding of GFP-xLBR$^{45-90}$ to importin β. GFP-xLBR$^{45-90}$ bound to importin β as efficiently as GFP-xLBR$^{1-210}$. (H) 1. GFP-xLBR$^{45-90}$ pulled down importin β from mitotic HeLa extract. 2. GFP-xLBR$^{45-90}$ pulled down importin β from *Xenopus* egg extract.

β. To map the C-terminal boundary, we constructed a series of C-terminally truncated xLBR proteins: GFP-xLBR$^{1-53}$, GFP-xLBR$^{1-81}$ and GFP-xLBR$^{1-90}$ (Fig. 5D) and carried out similar binding experiments. We found that GFP-xLBR$^{1-53}$ could not bind to importin β at all, whereas GFP-xLBR$^{1-90}$ could bind to importin β as effectively as GFP-xLBR$^{1-210}$ (Fig. 5E,F) suggesting that amino acids 45-90 are necessary and sufficient for binding to importin β. To test this possibility, we purified GFP-xLBR$^{45-90}$ and used it in the in vitro binding assay. As predicted, GFP-xLBR$^{45-90}$ could bind to importin β as efficiently as GFP-xLBR$^{1-210}$ (Fig. 5G). Furthermore, a pull-down assay with mitotic HeLa extract showed that GFP-xLBR$^{45-90}$ specifically and effectively pulled down importin β from the extract (Fig. 5H1). Likewise, GFP-xLBR$^{45-90}$ also specifically bound importin β in *Xenopus* egg extract (Fig. 5H2). These results demonstrated that xLBR binds to importin β through amino acids 45 to 90.

LBR binding to importin β is regulated by the nucleotide state of Ran and is importin α independent

The experiments above established that LBR associates with importin β through the direct binding of the N-terminal domain of LBR. We next investigate whether this interaction is regulated by the small GTPase Ran as Ran is known to affect importin β function. We first formed a LBR/importin β complex on agarose beads and then incubated the complex with or without Ran^{Q69L}-GTP or Ran^{T24N}-GDP and assayed for the release of LBR from importin β (Fig. 6A). We expected that if Ran directly regulated the binding of LBR to importin β, then incubation of the complex with Ran^{Q69L}-GTP would lead to the release of LBR from the importin β-bound agarose beads whereas buffer alone, or containing Ran^{T24N}-GDP, would have no effect on the binding. Incubation of this complex with 24 μM Ran^{Q69L}-GTP resulted in a nearly complete release of LBR from importin β (Fig. 6A, lanes 3, 4). Conversely incubation with buffer alone or 24 μM Ran^{T24N}-GDP did not release LBR from importin β (Fig. 6A, lanes 1, 2, lanes 5, 6). To ensure that this Ran effect is directly dependent on the Ran binding domain of importin β, we repeated the experiment with importin $β^{45-876}$, a deletion construct of importin β without the Ran binding domain (Kutay et al., 1997). Expectedly, RanQ69L-GTP failed to release LBR from importin $β^{45-876}$ (Fig. 6B, lanes 3, 4). These results demonstrated that Ran directly regulates the binding of LBR to importin β in a reconstituted system using purified proteins.

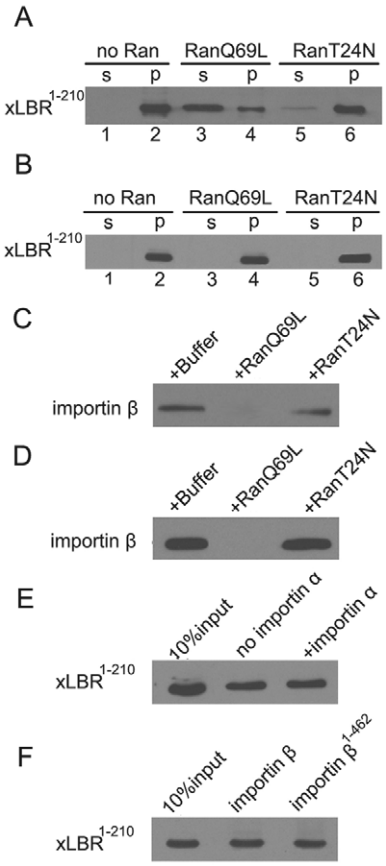

Fig. 6. LBR binding to importin β is regulated by the nucleotide state of Ran and is independent of importin α. (A) An in vitro assay of for the release of GST-importin β-bound His-$xLBR^{1-210}$ in the absence (no Ran, lanes 1 and 2) and presence of His-Ran^{Q69L}-GTP (RanQ69L, lanes 3 and 4) or His-Ran^{T24N}-GDP (RanT24N, lanes 5 and 6). The soluble (S) and pelleted (P) (bound to GST-importin β) proteins were analyzed for His-$xLBR^{1-210}$ with anti-His antibody. (B) An in vitro assay for the release of GST-importin $β^{45-876}$-bound His-$xLBR^{1-210}$ in the absence (no Ran, lanes 1 and 2) and presence of His-Ran^{Q69L}-GTP (RanQ69L, lanes 3 and 4) or His-Ran^{T24N}-GDP (RanT24N, lanes 5 and 6) as in A. (C,D) Pull-down assays of the endogenous importin β of mitotic HeLa cell extract (C) or *Xenopus* egg extract (D) by CNBr-activated Sepharose 4B-coated with His-$xLBR^{1-210}$ in the presence of buffer alone, His-Ran^{Q69L}-GTP or His-Ran^{T24N}-GDP. The LBR-bound importin β was probed with the anti-importin β antibody on western blots. (E) An in vitro binding assay of purified GST-importin β with His-$xLBR^{1-210}$ in the absence or presence of importin α. The LBR was probed with the anti-His antibody. (F) His-$xLBR^{1-210}$ binding assay with GST-importin β or GST-importin $β^{1-462}$ in vitro. His-$xLBR^{1-210}$ was probed with the anti-His antibody.

To demonstrate that the Ran regulation of LBR binding to importin β in a more physiological setting, we performed pull-down experiments using mitotic HeLa extract to which we added either Ran^{Q69L}-GTP or Ran^{T24N}-GDP. As shown in Fig. 6C, beads coated with 6His-LBR^{1-210} could not pull down importin β from mitotic HeLa in the presence of 24 μM Ran^{Q69L}-GTP. On the other hand, beads coated with 6His-LBR^{1-210} could pull down importin β from the extract without Ran or with Ran^{T24N}-GDP (Fig. 6C). Identical results were obtained when *Xenopus* egg extract was used (Fig. 6D). These results collectively demonstrated that Ran does regulate the interaction between LBR and importin β in a GDP-dependent manner.

As importin α is a partner of importin β for nuclear import, we wanted to know whether importin α played a role in the interaction between LBR and importin β. We carried out an in vitro binding assay in the presence or absence of purified importin α and observed similar levels of LBR-importin β interaction (Fig. 6E).The results indicated that importin α has no effect on the binding of LBR to importin β. To support this further, we performed an in vitro binding assay using purified importin $β^{1-462}$, a deletion construct of importin β lacking the importin α binding domain (Kutay et al., 1997), and found that importin $β^{1-462}$ could interact with LBR to a similar extent as full-length importin β (Fig. 6F). Thus, importin β binds to LBR in an importin α-independent manner.

The nuclear envelope precursor vesicles containing LBR are likely to participate in the nuclear envelope assembly through importin β

We previously used a mammalian mitotic cell extract to show that Ran GTPase and its partners drive NE assembly (Zhang and Clarke, 2001). Specifically Ran-coated Sepharose beads can organize NE assembly at the beads surface and importin β somehow links NE precursor vesicles to Ran through an unidentified mechanism (Zhang et al., 2002a). Since LBR binds directly to importin β, we reasoned that the NE precursor vesicles containing LBR may be recruited through the direct interaction with importin β to participate in NE assembly.

To investigate the direct recruitment of LBR-containing vesicles by importin β, we carried out cell-free NE assembly assays as it is very difficult to do so in intact cells (Zhang and Clarke, 2001). We established a HeLa cell line that stably expresses GFP-xLBR. Mitotic extract made from this cell line

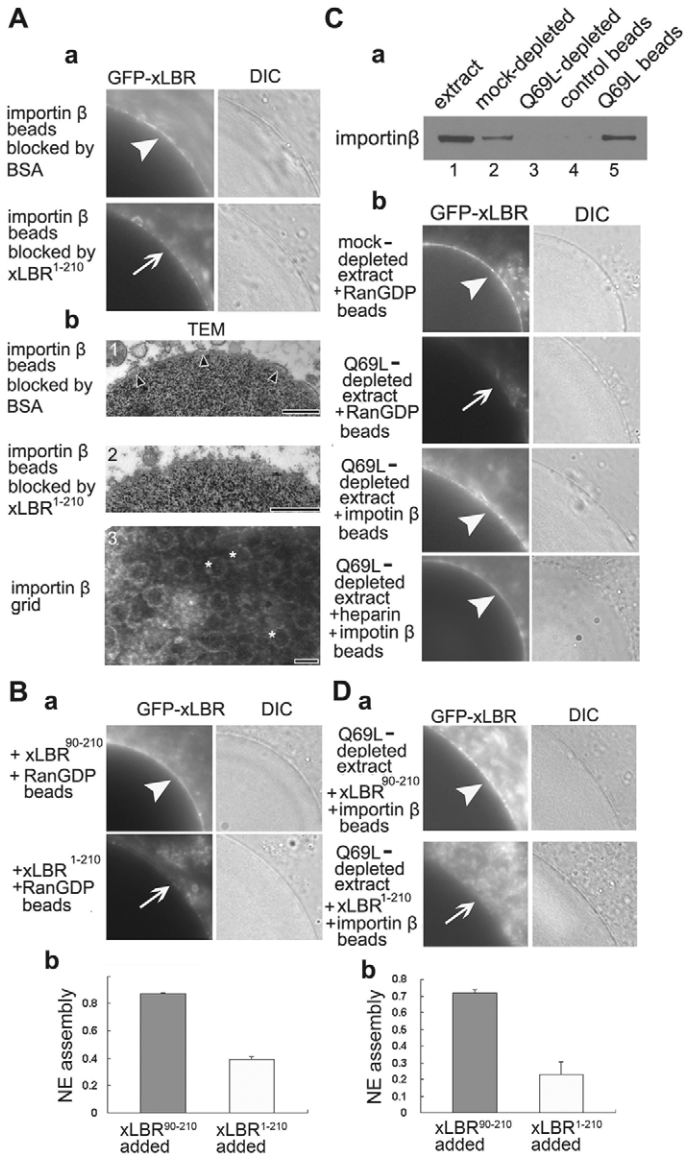

Fig. 7. LBR-containing NE precursor vesicles are recruited to participate in NE assembly through importin β. (Aa). Importin β-coated Sepharose beads were blocked in advance with BSA or xLBR$^{1-210}$ and used in the NE assembly assay in mitotic HeLa extract prepared from constitutive GFP-xLBR-expressing HeLa cells. Note that the incorporation of GFP-xLBR into NE could be blocked by xLBR$^{1-210}$ (indicated by arrow) but not by BSA (indicated by arrowhead). (Ab 1 and 2) Importin β-coated Dyna beads were blocked in advance with BSA (Ab 1) or xLBR$^{1-210}$ (Ab 2) and used in the NE assembly assay, followed by analysis with TEM. The double-layered NE (arrows) could be clearly seen. Note that xLBR$^{1-210}$ blocked the NE precursor vesicle recruitment and the NE assembly around the importin β-coated beads (Ab 2). Scale bar, 500 nm. (Ab 3) Negative staining of the NE with typical NPCs assembled on the surface of importin β-coated TEM grids. NPCs are indicated by asterisks. (Ba) RanGDP-coated beads induced NE assembly in mitotic HeLa extract prepared from constitutive GFP-xLBR-expressing HeLa cells in the presence of His-xLBR$^{90-210}$ or His-xLBR$^{1-210}$. The result showed that His-xLBR$^{1-210}$ (arrow) but not His-xLBR$^{90-210}$ (arrowhead) could specifically prevent recruitment of GFP-xLBR-bound NE precursor vesicles onto the RanGDP-beads to assemble the NE. (Bb) Statistical analysis of the NE assembly in the presence of His-xLBR$^{90-210}$ or His-xLBR$^{1-210}$. The data is shown as the mean percentage decorated beads plus the standard deviation. (C) NE assembly assay in the extract prepared from constitutive GFP-xLBR-expressing HeLa cells and depleted of importin β with Ran^{Q69L}. (Ca) Western blot analysis showed that more than 90% of the endogenous importin β in the extract was depleted by Ran^{Q69L}-GTP. The untreated extract (1), extract mock-depleted with control beads (2), extract depleted with Q69L beads (3), proteins bound to control (4) or Q69L (5) beads were subjected to Western blot analysis for importin β. (Cb) When NE assembly was induced with RanGDP-beads in the importin β-depleted HeLa extract, the NE assembly was efficiently blocked (indicated by arrow) compared with that in the mock-depleted extract (indicated by arrowhead). If importin β-coated beads were added to the importin β-depleted extract, the NE assembly could occur efficiently (indicated by arrowhead,). The addition of herparin to a final concentration of 0.5% did not influence the NE assembly around importin β-coated beads. (Da) When His-xLBR$^{90-210}$ or His-xLBR$^{1-210}$ was added to the importin β-depleted extract, the NE assembly around the added importin β-beads could only occur in the extract containing added His-xLBR$^{90-210}$ (indicated by arrow) but not His-xLBR$^{1-210}$ (indicated by arrowhead), indicating His-xLBR$^{1-210}$ prevented the access of the NE precursor vesicles to the importin β on the beads. (Db) Statistical analysis of the NE assembly in the presence of His-xLBR$^{90-210}$ or His-xLBR$^{1-210}$. The data is shown as the mean percentage (plus the standard deviation) of the beads decorated with the NE.

included abundant NE precursors containing GFP-xLBR, which could be easily observed using fluorescence microscopy as green fluorescent vesicles. First, we wanted to ascertain whether importin β could induce NE assembly around beads similar to Ran-coated beads as previously described (Zhang and Clarke, 2001). Indeed, importin β-coated beads non-specifically blocked with BSA recruited GFP-xLBR-containing vesicles, which resulted in a continuous NE-like membrane wrapping around the beads (Fig. 7Aa). To confirm that the NE around the importin β beads are induced double-layers, we carried out a TEM assay. We incubated importin β-coated Dyba beads with the HeLa extract to induce the NE assembly. Under the TEM, we clearly observed the double-layered membranes of the NE. To test if the NE had typical nuclear pore complexes (NPCs), we assembled the NE on the surface of the TEM grids coated with importin β. After negative staining, the grids were observed directly on the TEM. The result showed that importin β-coated grids efficiently recruited the NE precursor vesicles and induced the assembly of the NE, and that the NE possessed typical NPCs (Fig. 7Ab1,b2,b3). Next, we wished to know whether NE assembly around the importin β beads could be affected if importin β-LBR interaction is affected. For this purpose we added His-LBR$^{1-210}$ which can bind to importin β but lacks the transmembrane domains. If His-LBR$^{1-210}$ could block NE assembly by competing against GFP-xLBR-containing vesicles for binding to importin β, this would indicate that LBR is critical to the assembly of the NE through its interaction with importin β. Our results showed that importin β-coated beads blocked by His-xLBR$^{1-210}$ has less continuous and less GFP-xLBR-containing membrane around the beads than importin β-coated beads blocked with BSA (Fig. 7Aa). That is, the addition of the N-terminal domain of LBR was able to compete away importin β and therefore reduced the recruitment

efficiency of GFP-xLBR-containing vesicles. Although there was still considerable recruitment of GFP-xLBR-containing vesicles on the beads, it was probably due to the fact that the added LBR^{1-210} was not sufficient to completely compete away the binding sites of importin β for LBR. Alternatively, endogenous importin β might have bound and removed LBR^{1-210} from the importin β binding sites on the beads. To test these possibilities, we used RanGDP-coated beads to initiate the NE assembly. In such an experiment, we added purified His-LBR^{90-210} or His-LBR^{1-210} to the extract to a final concentration of ~10 μM and incubated for 30 minutes on ice. Then we added RanGDP-coated beads to induce NE assembly in the extract. RanGDP-coated beads induced nuclear assembly very well in the extract even in the presence of purified His-LBR^{90-210}, leading to the recruitment of GFP-xLBR vesicles to the beads to form a smooth membrane. By contrast, the GFP-xLBR-containing vesicles were not recruited to the beads in the extract with His-LBR^{1-210}, probably due to LBR^{1-210} competing with GFP-xLBR for binding to limited endogenous importin β (Fig. 7Ba,b). These data argue that LBR-containing vesicles are recruited to the Ran beads through importin β.

To further investigate the role of importin β in this process, we depleted importin β in the extract using Ran^{Q69L}-GTP (Zhang et al., 2002b). This procedure removed more than 90% of importin β from the HeLa cell extract (Fig. 7Ca) as well as other Ran-GTP binding proteins (Zhang et al., 2002b). The extract depleted of importin β failed to promote NE precursor recruitment and fusion to form a continuous membrane around the RanGDP-coated beads (Fig. 7Cb). However, this depleted extract was able to allow importin β-coated beads to recruit NE precursors to form the NE (Fig. 7Cb), indicating that NE precursors containing LBR can be recruited by direct interaction with importin β during the NE assembly and that importin β is the Ran-GTP binding protein sufficient for this recruitment. Moreover, addition of herparin, which can abolish weak ionic interactions, did not influence the NE assembly around importin β-coated beads (Fig. 7Cb). Furthermore, when we performed NE assembly assay using importin β-coated beads in the presence of purified His-$xLBR^{90-210}$ or His-$xLBR^{1-210}$, in this importin β-depleted system, we found that the excess purified $xLBR^{1-210}$ inhibited vesicles recruitment to the importin β-coated beads in the depleted extract whereas excess purified $xLBR^{90-210}$ had no effect (Fig. 7Da,b). Taken together, our data indicate that a direct interaction between LBR and importin β is crucial for the LBR-containing precursor vesicles to be recruited in the process of NE assembly.

Discussion

In this study, we (1) identified that LBR influences NE growth in a dose-dependent manner; (2) discovered a novel interaction between LBR and importin β; (3) demonstrated a role of this interaction in the recruitment of NE precursors during participate in NE assembly.

Lamin B receptor was identified in 1988 as an inner nuclear membrane protein (Worman et al., 1988). Here our data showed that overexpression of LBR in HeLa cells can lead to either NE invagination or large perinuclear aggregates. Ellenberg et al. previously reported that overexpression of human LBR^{1-238} induced NE invagination but not the perinuclear aggregates (Ellenberg et al., 1997). The difference was probably due to expression levels of the proteins since when we transfected HeLa cells with human LBR fused to GFP, perinuclear aggregates also formed (data not shown).

The transmembrane segments of LBR have high sequence identity to sterol reductase (Holmer et al., 1998; Silve et al., 1998). The finding that overexpression of the transmembrane segments of LBR led to formation of the perinuclear aggregates may indicate a new function for this domain. Wright et al. had reported that overexpression of HMG CoA reductase caused expansion of NE/ER membranes into structures termed 'karmellae' (Wright et al., 1988). But karmellae only wrap around the nucleus, do not bud off the nucleus, and form a distinct structure in the cytoplasm. However, the perinuclear aggregates we observed are swirled membrane stacks originating from, and coming off, the nucleus, forming an independent structure. Both LBR and HMG CoA reductase stimulated the membrane growth. In our opinion, the transmembrane segments of LBR may have the ability to change the curvature of the membrane facilitating the rounding into vesicle, thus allowing it to pinch off from the nucleus.

The hydrophilic N-terminal domain of LBR can interact with many proteins. First, it was found to interact with lamin B in a phosphorylation-dependent way (Appelbaum et al., 1990). Recently, it has been reported that LBR can bind to many other proteins such as heterochromatin protein 1 (HP1) (Ye and Worman, 1996) and HA95 (Martins et al., 2000). LBR even forms a complex including nuclear lamins, LBR kinase, p18 and p34 (Simos and Georgatos, 1992). Here we report a novel interaction between LBR and importin β and have identified the importin β binding domain on LBR (amino acids 45 to 90). Hydrophobic cluster analysis showed that the N terminus of LBR is composed of two globular domains separated by a hinge region ranging from amino acid 70 to 100 (Ye et al., 1997). The importin β binding site on LBR mainly falls into the hinge region, as well as part of the first globular domain, whereas HP1 binds distinctly to the second globular domain (Ye et al., 1997). Residues 45-90 of the *Xenopus* LBR amino acid sequence have high identity with residues 41 to 94 of human one. It is noteworthy that the arginine-serine (RS) repeat region lies within amino acid 81-90 in *Xenopus*, which regulates LBR binding to chromatin. Although the arginine-rich domains of HIV tat and Rev can bind directly with importin β (Truant and Cullen, 1999), our results show that the RS repeat abundant region of LBR is not sufficient for the binding of LBR to importin β. More recently, Blobel and colleagues reported that two novel INM proteins, Heh1p and Heh2p, are targeted to the INM in budding yeast through the importin α/β pathway (King et al., 2006). Amino acids 45-90 of LBR may represent a novel importin β-binding signal and the secondary and tertiary structure of this region may be critical for its function.

The RS repeat region of LBR is phosphorylated during interphase and mitosis and the binding of LBR to other proteins is regulated by this phosphorylation (Appelbaum et al., 1990; Nikolakaki et al., 1996; Nikolakaki et al., 1997). LBR maintains different phosphorylation states during interphase and mitosis (Nikolakaki et al., 1997) such that the mitotic phosphorylation state prevents LBR from binding to its binding partners, whereas at the onset of NE reassembly, upon entering interphase, LBR can once again bind to its partners because of changes in phosphorylation state (Nikolakaki et al., 1996). We

speculated that the interaction between LBR and importin β may also be regulated by phosphorylation. It will be of interest to determine in a future study whether LBR-importin β interaction is regulated similarly during cell cycle.

The interaction between importin β and Ran is modulated by the state of the bound nucleotide (GTP or GDP). RanGTP binding produces a substantial conformational change in full-length importin β (Lee et al., 2005). This conformational change within importin β leads to the release of importin β-binding proteins, such as importin α, from importin β (Lee et al., 2005; Blower et al., 2005; Gruss and Vernos, 2004; Ems-McClung et al., 2004). Here, we demonstrated that Ran GTPase modulated the interaction between LBR and importin β in the same way. This regulation is very important as Ran GTPase is required for NE assembly and importin β is crucial for the recruitment of NE precursor membranes.

The NE is disassembled when cells undergo mitosis, and at the end of mitosis the NE reassembles. The mechanism for NE assembly has been studied for decades, both in vivo and in cell free systems. Chaudhary and Courvalin reported that at the beginning of anaphase, the inner nuclear membrane-derived vesicles associate with chromatin first, whereas the pore membranes and the lamina assemble later, during telophase and cytokinesis (Chaudhary and Courvalin, 1993). As LBR is a well characterized integral protein located at the inner nuclear membrane, its role in NE reassembly has been studied extensively. In a cell free system, it was suggested that p56, a sea urchin LBR homologue, targeted membranes to chromatin and later anchored the membrane to the lamina (Collas et al., 1996). In addition, during the process of remodeling the sperm nucleus into a male pronucleus at fertilization, LBR-like protein targeted the membrane vesicles to the surface of chromatin (Collas et al., 1996). The fact that purified LBR can bind directly to chromatin fragments and decorates the surface of chromosomes in a distinctive binding pattern (Pyrpasopoulou et al., 1996) supports the model that LBR targets the membrane vesicles to the surface of chromatin during NE assembly.

However, in cell-free systems without chromatin, NE assembly occurs around the Ran GTPase- or importin β-coated Sepharose beads (Zhang and Clarke, 2000; Zhang et al., 2002a; Zhang et al., 2002b). Therefore, we propose that, in mediating NE precursor vesicles to bind to chromatin, LBR may first bind to a linker protein to mediate the membrane-chromatin attachment, as it was reported that the LBR-containing NE precursor vesicles can not bind directly to chromatin at the onset of nuclear assembly (Drummnond et al., 1999; Oke and Inoue, 2003). We propose that this linker protein is importin β, which is also very important for NE precursor vesicle recruitment (Zhang et al., 2002b).

In both NE formation and spindle assembly in *Xenopus* egg extracts and in tissue culture cells, Ran GTPase has been demonstrated to be a key regulator (Clarke and Zhang, 2001; Quimby and Dasso, 2003). Ran acts primarily through importin α and importin β, but the effect on importin β-binding effector proteins is likely to be different in the two processes (Zhang and Clarke, 2001). It is not yet known how the re-localization of Ran or changes in its molecular interactions at the end of mitosis are controlled, but binding of Ran to chromatin at telophase through importin β may increase the local concentration of Ran-GTP generated by RCC1, thereby promoting relatively low-affinity interactions with structural proteins involved in NE assembly, in which importin β probably plays a part (Zhang et al., 2002b). Hydrolysis of the GTP molecule on Ran is also required for membrane fusion during assembly of the NE, although the mechanism remains to be determined (Hetzer et al., 2000; Zhang and Clarke, 2000; Zhang et al., 2002a).

Here we present evidence that LBR acts by binding to importin β, which targets membrane vesicles that participate in NE assembly. We propose a model in which importin β targets LBR-containing NE membrane precursors to Ran-concentrated anaphase chromatin. Once binding with Ran-GTP on chromatin generated by RCC1, importin β immediately releases LBR-containing NE membrane precursors on the surface of chromatin. Ran-GTP hydrolysis would release importin β for another round of LBR targeting that would promote membrane vesicle fusion to form the NE (Zhang and Clarke, 2001).

It has been shown that Ran-regulated NE assembly is a conserved mechanism in all eukaryotes (Clarke and Zhang, 2001; Clarke and Zhang, 2004). LBR is also a conserved protein and located on the NE in all metazoans, *Drosophila* and yeast. No homologue of LBR has, so far, been found in plants, although the known plant sterol reductases share a sequence similarity to LBR in the membrane-targeting segments. However, when the human GFP-LBR$^{1\text{-}238}$ was expressed in tobacco plants, the fluorescence accumulated mainly at the NE, suggesting that plants may share common signals for NE targeting with animal and yeast cells, and/or that the LBR may have structural and functional plant homologues (Irons et al., 2003). The conservation of both the Ran regulation system and LBR function leads us to believe that LBR targeting of membrane to chromatin through importin β is a conserved mechanism.

Materials and Methods

Plasmids construction

Wild-type *Xenopus* LBR (NCBI Y17842) was cloned from a cDNA library of *Xenopus* oocytes (Clontech) and inserted into pET28a (Invitrogen) at the *Eco*RI and *Sal*I restriction sites. His-xLBR$^{1\text{-}210}$ was constructed by inserted the cDNA encoding amino acids 1-210 into the *Eco*RI/*Sal*I sites of pET28a and pEGFPC2. His-xLBR$^{90\text{-}210}$ and His-xLBR$^{211\text{-}621}$ were created similarly by cloning the cDNA encoding amino acids 90-210 or amino acids 211-621 into the *Eco*RI/*Sal*I sites of pEGFPC2. GFP-xLBR$^{309\text{-}621}$, containing amino acids 309-621, was cloned into the *Pst*I/*Sal*I sites of pEGFPC1. The xLBR$^{45\text{-}210}$, xLBR$^{53\text{-}210}$ and xLBR$^{81\text{-}210}$ cDNAs were subcloned into the *Eco*RI/*Sal*I sites of pET28a. pET28a-GFP was constructed by cloning EGFP from pEGFP into the *Nde*I/*Bam*HI sites of pET28a. The xLBR$^{1\text{-}210}$, xLBR$^{1\text{-}53}$, xLBR$^{1\text{-}81}$, xLBR$^{1\text{-}90}$ and xLBR$^{45\text{-}90}$ cDNAs were then subcloned into the *Eco*RI/*Sal*I sites of pET28a-EGFP. His-Ran^{Q69L} and His-Ran^{T24N} were also subcloned into the *Eco*RI/*Sal*I sites of pET28a. GST-importin β, GST-importin β$^{1\text{-}462}$ and GST-importin β$^{45\text{-}876}$ were constructed by cloning into the *Bam*HI site of pGEX-4T-1. Human importin α1 was cloned by RT-PCR and incorporated into the *Eco*RI/*Sal*I sites of pET28a.

Protein expression

Escherichia coli strain BL21(pLys) were transformed with either His-xLBR$^{1\text{-}210}$, His-xLBR$^{45\text{-}210}$, His-xLBR$^{53\text{-}210}$, His-xLBR$^{81\text{-}210}$, His-xLBR$^{90\text{-}210}$, His-importin α1, His-Ran^{Q69L} His-Ran^{T24N}, pET28a-EGFP, GFP-xLBR$^{1\text{-}210}$, GFP-xLBR$^{1\text{-}53}$, GFP-xLBR$^{1\text{-}81}$, GFP-xLBR$^{1\text{-}90}$, GFP-xLBR$^{45\text{-}90}$, GST-importin β, GST-importin β$^{1\text{-}462}$ or GST- importin β$^{45\text{-}876}$. To produce the recombinant truncated LBR, importin α1 and Ran proteins, cells were grown to an OD_{600} of ~1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM and the cells were incubated at 30°C for more than 5 hours to induce protein expression. To produce recombinant importin β, importin β$^{1\text{-}462}$ and importin β$^{45\text{-}876}$, the cell cultures were grown to an OD_{600} of ~0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.1 mM and the culture was incubated at 17°C for more than 6 hours to induce the protein expression. The bacterial cells were pelleted by centrifugation at 6,000 ***g*** for 10

minutes at 4°C. The proteins were purified with either Talon-Resin (BD Bioscience) or glutathione-Sepharose 4B (Pharmacia Biotech Inc.) following the manufacturer's instructions. Ran^{Q69L} and Ran^{T24N} were loaded with the appropriate nucleotide as described previously (Bischoff and Ponstingl, 1995). The purified protein was dialyzed in KHM buffer (78 mM KCl, 50 mM Hepes, pH 7.0, 4 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT) and stored in 10 μl aliquots at –70°C.

Cell culture and transfection

HeLa and XTC (*Xenopus* Tissue Culture) cell lines were used for transfection and immunofluorescence microscopy in this study. HeLa cells were grown in DMEM (Gibco) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. XTC cells were grown in 65% DMEM containing 10% fetal calf serum, sterile water, 100 units/ml penicillin, 100 μg/ml streptomycin. The cells, grown on 35-mm diameter Petri dishes, were transfected using the calcium precipitation method as described previously (Sambrook and Russell, 2001). Briefly, the precipitated plasmid DNA was left in the Petri dishes with the cells for 6 hours. Cells were then washed with PBS and grown for another 24-48 hours before direct observation and fixation for immunofluorescence and electron microscopy. The expression level was detected as the fluorescence intensity and analyzed using ImageJ software.

Immunofluorescence microscopy

Transfected cells were grown to 60% confluency on 35-mm diameter Petri dishes, washed three times with PBS, fixed with pre-cooled methanol for 5 minutes at room temperature and washed three times with PBS. Cells were then incubated with primary antibodies diluted in PBS containing 3% BSA (anti-nucleoporins monoclonal antibody mAb414 (Babco) diluted 1:500, anti-lamin B monoclonal antibody (Calbiochem) diluted 1:500, antibody against the Golgi marker GM130 (BD Bioscience) diluted 1:200, antibody against the ER marker calnexin (Santa Cruz) diluted 1:200, antibody against importin β (ABR) diluted 1:500 or antibody against $hLBR^{1-60}$ (generated by injecting rabbits) diluted 1:500, at room temperature for 1 hour. The cells were then washed five times in PBS, and incubated with secondary antibodies diluted in PBS containing 3% BSA [TRITC goat anti-mouse Ig (DAKO) diluted 1:200 or TRITC goat anti-rabbit Ig (DAKO) diluted 1:200] at room temperature for 45 minutes. The cells were then washed five times in PBS, drained and mounted in Mowiol (Sigma) containing 1 μg/ml 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.

HeLa cell extract preparation and NE assembly in vitro

Regular or constitutive GFP-xLBR-expressing mitotic HeLa cell extract was prepared as described previously (Zhang and Clarke, 2001). Briefly, HeLa cells stably grown in 20×175 cm^2 tissue culture flasks were synchronized by adding nocodazole (Sigma) to a final concentration of 100 ng/ml. After a further 12 hours of incubation, the mitotic cells were shaken off and collected by centrifugation at low speed. The cells were then washed three times at 4°C in KHM buffer followed by homogenization. The homogenates were centrifuged at 15,000 rpm in a bench-top centrifuge at 4°C. The supernatant was recovered and aprotinin was added to a final concentration of 10 μg/ml. Then glycerol was added to 5% and the extract was stored in liquid nitrogen. NE assembly around the Sepharose-beads was performed as described (Zhang and Clarke, 2001). Samples were removed and stained on a slide with 3,3′-dihexyloxacarbocyanine (DHCC) without fixation. If constitutive GFP-xLBR-expressing mitotic HeLa cell extract was used, the samples were observed directly without staining.

Transmission electron microscopy

Monolayer cells expressing GFP-xLBR were fixed with 1.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH 7.4), overnight at 4°C and washed 5 times with PB followed by the second fixation with 1% osmium tetroxide overnight at 4°C. The samples were embedded in Epon 812 and sectioned with a diamond knife. Sections were double-stained with uranyl acetate and lead citrate, and viewed on a transmission electron microscope TEM JEOL1010. Images were captured using a cooled charged-coupled device TEM camera AMT XR40.

Dyna beads (Dynal Biotech ASA) were washed three times with PB, mixed with GST-importin β (20 μm in KHM buffer: 78 mM KCl, 50 mM Hepes, pH 7.0, 4 mM $MgCl_2$, 2 mM EGTA, and 1 mM DTT) or BSA as control, and rotated gently at 4°C for 24 hours. The mixtures were added with 0.5% BSA (final concentration) and incubated for an additional 30 minutes to block the reaction. The Dyna beads were washed three times with PB and resuspended in KHM buffer. 4×10^5 beads in 1 μl were incubated with 50 μl mitotic HeLa cell extract at 23°C for 2 hours to induce NE assembly around the beads. The samples were removed and double-fixed with glutaraldehyde [2.5% (v/v) in 0.1 M PB] and OsO_4 (1.5% in 0.1 M PB). After dehydration in a graded series of acetone (15 minutes each), the samples were embedded in the Spur resin and sectioned. Sections were double-stained with uranyl acetate and lead citrate, and viewed on the TEM JEOL1010. Images were captured using the TEM camera AMT XR40.

Gel electrophoresis and immunoblotting

After being resolved 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 hour 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% non-fat milk for 1 hour at room temperature and probed with anti-His monoclonal antibody (Santa Cruz) diluted 1:1,000 in TTBS with 5% nonfat milk or probed with anti-importin β monoclonal antibody (Transduction) diluted 1:1,000 in TTBS with 5% non-fat milk overnight at 4°C. The filters were then washed three times and blocked again for 30 minutes in TTBS containing 5% non-fat milk and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson) diluted 1:1,000 in TTBS with 5% non-fat milk for 1 hour at room temperature. After final washes in TTBS, the filters were developed for visualization by enhanced chemiluminescence (Sigma) and X-ray films.

Binding assay with mitotic HeLa extract and *Xenopus* crude egg extract

HeLa mitotic extract was prepared as described above and *Xenopus* crude egg extract was prepared as described (Hartl et al., 1994). Briefly, eggs were dejellied and rinsed with extraction buffer (50 mM Hepes-KOH, pH 7.4, 50 mM KCl and 2 mM $MgCl_2$), and centrifuged at 12,000 ***g*** for 20 minutes two times, the supernatant was removed as the crude egg extract. For binding assay, CNBr-activated Sepharose 4B beads (Amersham Biosciences) coated with equal amounts (~4 μg) of GFP, $GFP\text{-}LBR^{1\text{-}210}$ or $GFP\text{-}LBR^{45\text{-}90}$ were incubated in 20 μl mitotic HeLa extract or *Xenopus* egg extract diluted 10-fold in ice-cold dilution buffer [20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.1% (v/v) Triton X-100, and 10% (v/v) glycerol] for 2 hours at 4°C with continuous gentle agitation. The beads were recovered by slow speed centrifugation (2,600 ***g***) and washed four times with the dilution buffer. Proteins were eluted with SDS-PAGE loading buffer and analyzed by western immunoblotting using the indicated antibodies.

In vitro binding assay

In binding experiments, about 3 μg of the various purified xLBR fragments were added to 400 μl binding buffer (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0 and 0.1% NP-40), then incubated with 10 μl of glutathione-Sepharose (Pharmacia Biotech) coupled with 6.0 μg glutathione S-transferase (GST), GST-importin β, GST-importin $\beta^{45\text{-}876}$ or GST-importin $\beta^{1\text{-}462}$. The suspensions were incubated at 4°C with rotation for 2 hours. After incubation, the Sepharose was washed five times with binding buffer, and the bound proteins were eluted with SDS sample buffer. In assays using different salt concentrations, the binding buffers contained the indicated concentrations of salt. Meanwhile, after incubation a small portion of the beads was taken out for direct fluorescence microscopic analysis.

We gratefully thank all the other members in our laboratory for valuable comments and discussion and Shilai Bao (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences) for anti-GP130 antibody. This work was supported by funds of the National Natural Science Foundation of China (NNSFC) (30421004, 30225016 and 30330200), of the Ministry of Science and Technology of China (2004CB720003 and 2006CB0D0100), and of the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Ministry of Education of P.R. China.

References

Appelbaum, J., Blobel, G. and Georgatos, S. D. (1990). In vivo phosphorylation of lamin B receptor. Binding of lamin B to its membrane receptor is affected by phosphorylation. *J. Biol. Chem.* **265**, 4181-4184.

Askjaer, P., Galy, V., Hannak, E. and Mattaj, I. W. (2002). Ran GTPase cycle and importin alpha and beta are essential for spinde formation and nuclear envelope assembly in *Caenorhabditis elegans* embryos. *Mol. Biol. Cell* **13**, 4355-4370.

Bayliss, R., Littlewood, T. and Stewart, M. (2000). Structural basis for the interaction between FXFG nucleoporin repeats and importin beta in nuclear trafficking. *Cell* **102**, 99-108.

Bischoff, F. R. and Ponstingl, H. (1995). Catalysis of guanine nucleotide exchange of Ran by RCC1 and stimulation of hydrolysis of Ran-bound GTP by Ran-GAP1. *Meth. Enzymol.* **257**, 135-144.

Blower, M. D., Nachury, M., Heald, R. and Weis, K. (2005). A Rea1-containing ribonucleoprotein complex is required for mitotic spindle assembly. *Cell* **121**, 223-234.

Chaudhary, N. and Courvalin, J. C. (1993). Stepwise reassembly of the nuclear envelope at the end of mitosis. *J. Cell Biol.* **122**, 295-306.

Clarke, P. R. and Zhang, C. (2001). Ran GTPase: a master regulator of nuclear structure and function during the eukaryotic cell division cycle? *Trends Cell Biol.* **11**, 366-371.

Clarke, P. R. and Zhang, C. (2004). Spatial and temporal control of nuclear envelope assembly by Ran GTPase. *Symp. Soc. Exp. Biol.* **56**, 193-204.

Collas, P., Courvalin, J. C. and Poccia, D. (1996). Targeting of membranes to sea urchin

sperm chromatin is mediated by a lamin B receptor-like integral membrane protein. *J. Cell Biol.* **135**, 1715-1725.

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

Drummond, S., Ferrigno, P., Lyon, C., Murphy, J., Goldberg, M., Allen, T., Smythe, C. and Hutchison, C. J. (1999). Temporal differences in the appearance of NEP-B78 and an LBR-like protein during *Xenopus* nuclear envelope reassembly reflect the ordered recruitment of functionally discrete vesicle types. *J. Cell Biol.* **144**, 225-240.

Duband-Goulet, I. and Courvalin, J. C. (2000). Inner nuclear membrane protein LBR preferentially interacts with DNA secondary structures and nucleosomal linker. *Biochemistry* **39**, 6483-6488.

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

Ems-McClung, S. C., Zheng, Y. and Walczak, C. E. (2004). Importin α/β and Ran-GTP regulate XCTK2 microtubule binding through a bipartite nulcear localization signal. *Mol. Biol. Cell* **15**, 46-57.

Gajewski, A. and Krohne, G. (1999). Subcellular distribution of the *Xenopus* p58/lamin B receptor in oocytes and eggs. *J. Cell Sci.* **112**, 2583-2596.

Gant, T. M. and Wilson, K. L. (1997). Nuclear assembly. *Annu. Rev. Cell Dev. Biol.* **13**, 669-695.

Georgatos, S. D. (2001). The inner nuclear membrane: simple, or very complex? *EMBO J.* **20**, 2989-2994.

Gruss, O. J. and Vernos, I. (2004). The mechanism of spindle assembly: functions of Ran and its target TPX2. *J. Cell Biol.* **166**, 949-955.

Haraguchi, T., Koujin, T., Hayakawa, T., Kaneda, T., Tsutsumi, C., Imamoto, N., Akazawa, C, Sukegawa, J., Yoneda, Y. and Hiraoka, Y. (2000). Live fluorescence imaging reveals early recruitment of emerin, LBR, RanBP2, and Nup153 to reforming functional nuclear envelopes. *J. Cell Sci.* **113**, 779-794.

Hartl, P., Olson, E., Dang, T. and Forbes, D. J. (1994). Nuclear assembly with lambda DNA in fractionated *Xenopus* egg extract: an unexpected role for glycogen in formation of a higher order chromatin intermediate. *J. Cell Biol.* **124**, 235-248.

Hetzer, M., Bilbao-Cortes, D., Walther, T. C., Gruss, O. J. and Mattaj, I. W. (2000). GTP hydrolysis by Ran is required for nuclear envelope assembly. *Mol. Cell* **5**, 1013-1024.

Holmer, L., Pezhman, A. and Worman, H. J. (1998). The human lamin B receptor/sterol reductase multigene family. *Genomics* **54**, 469-476.

Irons, S. L., Evans, D. E. and Brandizzi, F. (2003). The first 238 amino acids of the human lamin B receptor are targeted to the nuclear envelope in plants. *J. Exp. Bot.* **54**, 943-950.

Kawahire, S., Takeuchi, M., Gohshi, T., Sasagawa, S., Shimada, M., Takahashi, M., Abe, T. K., Ueda, T., Kuwano, R., Hikawa, A. et al. (1997). cDNA cloning of nuclear localization signal binding protein NBP60, a rat homologue of lamin B receptor, and identification of binding sites of human lamin B receptor for nuclear localization signals and chromatin. *J. Biochem.* **121**, 881-889.

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

Kutay, U., Izaurralde, E., Bischoff, F. R., Mattajm, I. W. and Gorlich, D. (1997). Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. *EMBO J.* **16**, 1153-1163.

Lee, S. J., Matsuura, Y., Liu, S. M. and Stewart, M. (2005). Structural basis for nuclear import complex dissociation by RanGTP. *Nature* **435**, 693-696.

Makatsori, D., Kourmouli, N., Polioudaki, H., Shultz, L. D., McLean, K., Theodoropoulos, P. A., Singh, P. B. and Georgatos, S. D. (2004). The inner nuclear membrane protein lamin B receptor forms distinct microdomains and links epigenetically marked chromatin to the nuclear envelope. *J. Biol. Chem.* **279**, 25567-2573.

Martins, S. B., Eide, T., Steen, R. L., Jahnsen, T., Skålhegg, B. S. and Collas, P. (2000). HA95 is a protein of the chromatin and nuclear matrix regulating nuclear envelope dynamics. *J. Cell Sci.* **113**, 3703-3713.

Meier, J. and Georgatos, S. D. (1994). Type B lamins remain associated with the integral nuclear envelope protein p58 during mitosis: implications for nuclear reassembly. *EMBO J.* **13**, 1888-1898.

Nikolakaki, E., Simos, G., Georgatos, S. D. and Giannakouros, T. (1996). A nuclear envelope-associated kinase phosphorylates arginine-serine motifs and modulates interactions between the lamin B receptor and other nuclear proteins. *J. Biol. Chem.* **271**, 8365-8372.

Nikolakaki, E., Meier, J., Simos, G., Georgatos, S. D. and Giannakouros, T. (1997). Mitotic phosphorylation of the lamin B receptor by a serine/arginine kinase and p34cdc2. *J. Biol. Chem.* **272**, 6208-6213.

Oke, Y. and Inoue, A. (2003). The interaction of two classed of nuclear vesicles is induced by the dissociation of soluble proteins from one class of vesicles. *Biol. Cell* **95**, 87-97.

Pyrpasopoulou, A., Meier, J., Maison, C., Simos, G. and Georgatos, S. D. (1996). The lamin B receptor (LBR) provides essential chromatin docking sites at the nuclear envelope. *EMBO J.* **15**, 7108-7119.

Quimby, B. B. and Dasso, M. (2003). The small GTPase Ran: interpreting the signs. *Curr. Opin. Cell Biol.* **15**, 338-344.

Sambrook, J. and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (3rd edn). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Shah, S., Tugendreich, S. and Forbes, D. (1998). Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr. *J. Cell Biol.* **141**, 31-49.

Shultz, L. D., Lyons, B. L., Burzenski, L. M., Gott, B., Samuels, R., Schweitzer, P. A., Dreger, C., Herrmann, H., Kalscheuer, V., Olins, A. L. et al. (2003). Mutations at the mouse ichthyosis locus are within the lamin B receptor gene: a single gene model for human Pelger-Huet anomaly. *Hum. Mol. Genet.* **12**, 61-69.

Silve, S., Dupuy, P. H., Ferrara, P. and Loison, G. (1998). Human lamin B receptor exhibits sterol C14-reductase activity in *Saccharomyces cerevisiae. Biochim. Biophys. Acta* **1392**, 233-244.

Simos, G. and Georgatos, S. D. (1992). The inner nuclear membrane protein p58 associates in vivo with a p58 kinase and the nuclear lamins. *EMBO J.* **11**, 4027-4036.

Smith, S. and Blobel, G. (1993). The first membrane spanning region of the lamin B receptor is sufficient for sorting to the inner nuclear membrane. *J. Cell Biol.* **120**, 631-637.

Soullam, B. and Worman, H. J. (1993). The amino-terminal domain of the lamin B receptor is a nuclear envelope targeting signal. *J. Cell Biol.* **120**, 1093-1100.

Takano, M., Takeuchi, M., Ito, H., Furukawa, K., Sugimoto, K., Omata, S. and Horigome, T. (2002). The binding of lamin B receptor to chromatin is regulated by phosphorylation in the RS region. *Eur. J. Biochem.* **269**, 943-953.

Truant, R. and Cullen, B. R. (1999). The arginine-rich domains present in human immnuodeficiency virus type 1 tat and rev function as direct importin beta-dependent nuclear localization signals. *Mol. Cell. Biol.* **19**, 1210-1217.

Wagner, N., Weber, D., Seitz, S. and Krohne, G. (2004). The lamin B receptor of Drosophila melanogaster. *J. Cell Sci.* **117**, 2015-2028.

Walther, T. C., Askjaer, P., Gentzel, M., Habermann, A., Griffiths, G., Wilm, M., Mattaj, I. W. and Hetzer, M. (2003). RanGTP mediates nuclear pore complex assembly. *Nature* **424**, 689-694.

Waterham, H. R., Koster, J., Mooyer, P., Noort Gv, G., Kelley, R. I., Wilcox, W. R., Wanders, R. J., Hennekam, R. C. and Oosterwijk, J. C. (2003). Autosomal recessive HEM/Greenberg skeletal dysplasia is caused by 3 beta-hydroxysterol delta 14-reductase deficiency due to mutations in the lamin B receptor gene. *Am. J. Hum. Genet.* **72**, 1013-1017.

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

Worman, H. J., Evans, C. D. and Blobel, G. (1990). The lamin B receptor of the nuclear envelope inner membrane: a polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* **111**, 1535-1542.

Wright, R., Basson, M., D'Ari, L. and Rine, J. (1988). Increased amounts of HMG-CoA reductase induce "karmellae": a proliferation of stacked membrane pairs surrounding the yeast nucleus. *J. Cell Biol.* **107**, 101-114.

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

Ye, Q. and Worman, H. J. (1996). Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. *J. Biol. Chem.* **271**, 14653-14656.

Ye, Q., Callebaut, I., Pezhman, A., Courvalin, J. C. and Worman, H. J. (1997). Domain-specific interactions of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. *J. Biol. Chem.* **272**, 14983-14989.

Zhang, C. and Clarke, P. R. (2000). Chromatin-independent nuclear envelope assembly induced by Ran GTPase in *Xenopus* egg extracts. *Science* **288**, 1429-1432.

Zhang, C. and Clarke, P. R. (2001). Roles of Ran-GTP and Ran-GDP in precursor vesicle recruitment and fusion during nuclear envelope assembly in a human cell-free system. *Curr. Biol.* **11**, 208-212.

Zhang, C., Goldberg, M. W., Moore, W. J., Allen, T. D. and Clarke, P. R. (2002a). Concentration of Ran on chromatin induces decondensation, nuclear envelope formation and nuclear pore complex assembly. *Eur. J. Cell Biol.* **81**, 623-633.

Zhang, C., Hutchins, J. R., Muhlhausser, P., Kutay, U. and Clarke, P. R. (2002b). Role of importin-beta in the control of nuclear envelope assembly by Ran. *Curr. Biol.* **12**, 498-502.