Postmitotic annulate lamellae assembly contributes to nuclear envelope reconstitution in daughter cells

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In higher eukaryotic cells, the nuclear envelope (NE) is composed of double nuclear membranes studded with nuclear pore complexes (NPCs) and undergoes dynamic disassembly and reassembly during the cell cycle. However, how the NE and NPC reassemble remains largely unclear. Here, using HeLa, HEK293, and Drosophila cells, along with immunofluorescence microscopy and transmission EM methods, we found that postmitotic annulate lamellae (AL) assembly contributes to NE and NPC assembly. We observed that the AL are parallel membrane-pair stacks and possess regularly spaced AL pore complexes (ALPCs) that are morphologically similar to the NPCs. We found that the AL assemble in the cytoplasm during mitotic exit simultaneously with NE re-formation in daughter cells. Then, the assembled AL either bound the decondensing chromatin to directly transform into the NE or bound and fused with the outer nuclear membrane to join the assembling NE. The AL did not colocalize with sheet and tubular endoplasmic reticulum (ER) marker proteins on the ER or the lamin B receptor–localized membrane in the cytoplasm, suggesting that postmitotic AL assembly occurs independently of the chromatin and ER. Collectively, our results indicate that postmitotic AL assembly is a common cellular event and an intermediate step in NE and NPC assembly and in NE expansion in higher eukaryotic cells.

Proper cell proliferation is essential for cell life, individual development, and health. In cell proliferation, the nuclear periphery domains, including the nuclear envelope (NE), nuclear pore complex (NPC), and nuclear lamina (NL), undergo dynamic disassembly processes during mitotic entry and reassembly at the end of mitosis (1, 2). The NE is a double-layered membrane that contains many NPCs that are mainly composed of nucleoporins (Nups). The NE hosts dozens of specially localized NE proteins in its inner nuclear membrane, and its outer nuclear membrane (ONM) is continuous with the endoplasmic reticulum (ER). When the cell enters mitosis, the NE is retracted into the ER or fragmented into membrane vesicles, whereas the NPCs disassemble into soluble Nups (3, 4). At the end of mitosis, the NE, NL, and NPCs reassemble around the separated daughter chromatids using their previously disassembled components (5–8).

NE, NL, and NPC assembly can be mimicked in Xenopus egg extracts to faithfully mimic cell cycle regulation (9, 10). The NE, NL, and NPCs can assemble around chromatin, purified DNA, and even artificial beads coated with specified proteins (11–20). Increasing evidence indicates that NE and NPC assembly in cells and Xenopus egg extracts consists of stepwise loading processes with different membranes and Nups (21–23). Similar to the process in cells, the NE assembled in Xenopus egg extracts expands, and the NPC number increases to meet the needs of nuclear growth (10, 24–27).

Annulate lamellae (AL) are parallel membrane pair stacks that are found in almost all cell types. They possess regularly spaced AL pore complexes (ALPCs) that are morphologically similar to NPCs (28–30). Although the AL have an antigenically distinct molecular make-up from that of NPCs and other subcellular membranes (30, 31), many ALPC components can be recognized by antibodies against NPC components (32). Furthermore, overexpressing Nups may induce AL formation in mammalian cells (33), indicating that both NPCs and ALPCs share similar components. The AL have been proposed to provide a store of the NPCs in rapidly dividing cells during early embryogenesis, but the precise function of the AL is controversial (34, 35). In particular, when the AL assemble and the fate of AL are unclear (36).

More recently, the AL were found to play a role in the increase in NPC numbers in Drosophila syncytial embryos by assembling NPC scaffolds and then fusing them with the interphase NE (29). Hampool et al. also found that AL assembled in syncytial embryos and inserted into the NE during interphase at the end of mitosis. Accordingly, the authors developed a topological model in which NE openings were critical for AL uptake. However, this insertion operates only in early embryos before gastrulation (29), and the AL in other cells remain a functional mystery.

In this work, we found that the AL efficiently assembled in the cytoplasm at the end of mitosis in both cultured cells and developing Drosophila somatic cells. We reveal that the AL...
either directly bound the chromatin to contribute to NE and NPC assembly or fused with the outer nuclear membrane to contribute to NE assembly, NPC number increases, and NE expansion.

**Results**

**Nup-containing particles spontaneously assembled in the cytoplasm of all cells during mitotic exit**

To deeply investigate the mechanism of NE and NPC assembly or fused with the outer nuclear membrane to contribute to NE assembly, NPC number increases, and NE expansion.

**Nup-containing particles spontaneously assembled in the cytoplasm of all cells during mitotic exit**

Figure 1. NE- and Nup-containing particles simultaneously assemble at the end of mitosis. A and B, HeLa cells were immunostained with antibodies against Nups (mAb414), LBR, and calnexin. Note the presence of many Nup-containing particles (indicated by arrows) in the cytoplasm of early and late G₁-phase cells and that the number of Nup-containing particles decreases during nuclear growth. Additionally, the Nup-containing particles do not localize with either the LBR- or calnexin-containing ER. Bars, 10 μm. C and D, HeLa cells transiently expressing GFP–Nups were immunostained with mAb414. M, metaphase; A/T, anaphase/telophase; EG₁, early G₁; LG₁, late G₁; and LI, late interphase. Note that GFP–Nup-overexpressing early G₁ cells possess more Nup-containing particles (green or red dots in the cytoplasm) than cells in late G₁ and other phases. Bar, 10 μm.
calized with neither the LBR- nor calnexin-containing ER except in the NE (Figs. 1, A and B, S1).

To investigate the nature of these particles, we expressed GFP-tagged exogenous Nups and communostained endogenous Nups in cells. We observed that both exogenous and endogenous Nups colocalized on the particles in addition to the NE in all dividing cells (Fig. 1, C and D). Furthermore, regardless of whether the cells had high, low, or no exogenous Nup expression, the amount of Nup–containing particles was comparable in both exogenous and nonexogenous Nup–expressing cells (Fig. 1D), suggesting that the particle assembly was not due to exogenous Nup overexpression.

Taken together, these results indicate that Nup-containing particles assemble at the end of mitosis in a manner that is relatively independent of the ER.

Nup-containing particles were actually the AL contributing to NE assembly

To trace the assembly process and fate of these particles, we generated cell lines stably expressing GFP-tagged Nups and performed time-lapse microscopy. We found that the GFP–Nups incorporated into the NPCs in interphase and dispersed into the cytoplasm in mitosis (Fig. 2A, cell 1 (C1) and cell 2 (C2); Fig. S2; Movies S1 and S2). Interestingly, when these cells divided, the GFP–Nup-containing particles simultaneously assembled near the separated chromatids and gradually assembled into the assembling NE (Fig. 2A). By comparing fluorescence intensity of GFP–Nups in 30 live HeLa cells at the end of mitosis, we calculated the ratio of these cytoplasmic particles to the NE. We found that the ratio was roughly 4.8 to 1 and that more than 40% of these AL joined into the assembling NE within 4 h, whereas most if not all of the rest joined the NE in later phases. We revealed that less than 20% of G2 cells were occasionally with AL, and these AL disassembled into the dispersed Nups in the cytoplasm during mitotic re-entry. Through immunostaining of the Nups and 3D analysis, we observed direct binding of the Nup-containing particles to the chromatin during NE assembly (Fig. 2B; Movie S3). Fast live-cell imaging of GFP–Nup107 also revealed cytoplasmic AL assembly and recruitment to the separating chromatids at anaphase (Fig. 2C; Movie S4). By using transmission EM (TEM), we observed that these particles were actually AL and that these AL could directly bind to the chromatin to transform into the NE (Fig. 2D). By coexpressing Nups with LBR or calnexin, we confirmed that AL assembly was relatively independent of cytoplasmic LBR- and calnexin-containing membranes (Movies S5 and S6). We also colabeled Nup107 with two other ER markers (sheet ER marker Climp63 and tubule ER marker Reep5) and found that Nup107-containing AL did not colocalize well with either the Climp63-containing ER or Reep5-containing ER (Fig. S4, A and B). We expressed mCherry–Sec61–β in GFP–Nup107 stably-expressing HeLa cells prior to performing live-cell imaging, and we observed that the GFP-containing AL did not colocalize with the mCherry–Sec61–β-containing ER (Fig. S4C). Using structural illumination microscopy for super-resolution live-cell imaging, we observed that some of the AL contacted the ER, which was consistent with a previous report (29), whereas others did not (Fig. S4D).

Taken together, these results demonstrate that in addition to the ER and previously disassembled NE membrane vesicles, the membrane and ALPCs of the AL are also sources of the NE and NPCs and that AL assembly and ER reconstruction are relatively independent of each other.

AL can transform into the NE through fusion with the ONM

Because the AL can bind chromatin and participate in NE assembly during mitotic exit, we investigated the fate of the AL. Through time-lapse microscopy, we observed that the AL directly bound and fused into the assembling NE (Fig. 3A; Movie S7). Under TEM, we observed that the AL varied in size from larger structures that contained several piled lamellae to smaller structures that contained only a small piece of lamella (Fig. 3B). We revealed that the smaller AL fused with the ONM through its margin membrane (Fig. 3C). Through series sectioning, we found that the larger AL fused with the NE through the lamella nearest to the NE (Fig. 3D). By correlative light and EM using a stable GFP–Nup107-expressing cell line, we also revealed the fusion sites (Fig. S3, E–H; Movie S8). However, we did not observe any NEs that created holes first and then recruited AL to bind the chromatin, indicating that AL could only join the intact NE after assembly through fusion. Taken together, these results demonstrate that when the assembling NE is sealed, the AL can still join the NE to contribute to rapid NE assembly and expansion and that the AL fuses with the NE through their membranes.

AL assembly contributes to NE assembly and expansion during Drosophila development

Because AL assembly was found to contribute to NE assembly in Drosophila syncyrtial embryos (29, 37), we took advantage of this animal model with fast development using time-lapse microscopy. First, we recorded syncyrtial embryos of Drosophila that persistently expressed GFP–Nup107. Consistent with a previous report (29), we observed that both the AL and NE in interphase syncytial embryos disassembled during mitotic entry and reassembled at the end of mitosis and that the AL inserted into the interphase NE during nuclear growth (Fig. 4A; Movie S9). We also found that the AL could directly bind chromatin to transform into the NE (Fig. 4A; Movie S9). Next, through recordings of cellular blastoderm embryos after 13 synchronized mitoses, we found that the AL also assembled and contributed to NE assembly and expansion by directly binding the chromatin and fusing with the intact assembling NE at the end of mitosis and G1 phase (Fig. 4B; Movie S10). With great interest, we investigated whether AL assembly also occurred in individuals in the late developmental stage. We dissected third instar larval brains of Drosophila that constitutively expressed GFP–Nup107 and recorded the cell division process of neuroblast cells. As expected, we found that postmitotic AL assembly occurred in individuals at this stage and that the assembled AL contributed to NE assembly and expansion (Fig. 4C; Movie S11). From recordings of Drosophila embryos and larvae that constitutively expressed RFP–Nup107 rather than GFP–Nup107, we obtained the same
results as the experiments using GFP-expressing individuals (Fig. 4D; Movie S12).

In summary, we conclude that postmitotic AL assembly occurs in all proliferating cells and that the assembled AL then transform into the NE through either direct binding to the chromatin during NE assembly or fusing into the existing assembling NE for expansion. This mechanism is universal for NE reconstitution in all proliferating cells.
Figure 3. AL can fuse with the NE to contribute to NE assembly and expansion. A, fusion of the AL with the NE in HeLa cells stably expressing GFP–Nup35. Arrows indicate gradual fusion of the AL into the NE. Bar, 10 μm. B, TEM showing a portion of a G1 phase cell with the AL. Nu, nucleus; Mit, mitochondrion. Box 1 is zoomed into box 1’. The black arrow indicates a small AL. Bar in the main image is 1 μm and in box 1’ is 500 nm. C, TEM showing an AL that fuses with the NE elucidated by a cartoon (indicated by the white arrow). The black arrows indicate a small AL, and the black arrowheads indicate the NPCs. Nu, nucleus. Bar, 300 nm. D, TEM serial sections A and B with elucidation cartoons of the white boxed areas to show fusion of the AL with the outer nuclear membrane. Arrows indicate the fusion sites. Bar, 500 nm. E and F, correlative light and EM revealing fusion sites of the AL with the NE. E, HeLa cells with GFP–Nup107 expression. Bar, 10 μm. F, focused ion beam–scanning electron microscopy (FIB-SEM) of the boxed area in E. Bar, 2 μm. G, zoomed image of the boxed area in F. Black arrow indicates one fusion site of the AL with the ONM. Bar, 500 nm. H, 3D reconstruction model showing the structural relationship between the NE and AL. NE is in green; AL is in red, and a membrane vesicle is in blue. Bar, 500 nm.
Discussion

In this work, we studied the mechanism of NE assembly and expansion in higher eukaryotic cells. We demonstrate that both the NE and AL assemble simultaneously at the end of mitosis and that postmitotic AL assembly contributes to NE assembly and expansion by serving as an intermediate step in NE reconstitution. The AL either directly bind with the chromatin to transform into the NE or fuse with the assembling NE to contribute to NE assembly.

The AL are parallel membrane stacks that contain NPC-like ALPCs and are often observed in a variety of cell types in both animals and plants (28, 29, 37). This work clearly determined the timing of AL assembly and the AL fate mainly by taking advantage of time-lapse microscopy and hence answered long-lasting questions concerning why fast-proliferating cells assemble AL, the functions of the AL, and how the assembling daughter nuclei rapidly expand their NEs.

How cells assemble the AL is another long-persisting question. This work found that AL assembly is relatively independent of cytoplasmic LBR- or calnexin-containing membranes. AL assembly more likely initiates by assembling a small lamellar piece that contains only a few ALPCs using a currently undetermined membrane and some Nups. Under TEM, we did observe small AL with one or two ALPCs in early G1 cells (Fig. 3B). A number of the small lamellar pieces combine to form the larger AL. Both small and large AL can directly bind chromatin to transform into the NE. Clearly, chromatin is not essential for AL assembly. Instead, chromatin decondensation and AL assembly are probably parallel events, and the AL transform into the NE only when they have bound to chromatin, which allows them to transform into the NE on the decondensing chromatin. If the AL cannot efficiently bind chromatin before the NE is sealing up, they can still insert into the NE through fusion.

However, at present, we do not know the mechanism underlying membrane fusion of the AL with the NE, although we clearly observed the fusion site of the AL with the outer nuclear membrane. Beck and co-workers (29) have proposed a model for insertion of ALPCs into the NE. However, this model is based on connection of the NE with the ER sheet and on the fact that this ER sheet is thought to be the location of ALPC assembly. Here, we find that both small and large AL are assembled relatively independently of the ER sheet. Moreover, we find that AL assembly and fusion with the NE not only occur in early Drosophila embryos before gastrulation but also in cellular embryos and late developing individuals. Therefore, we propose that postmitotic AL assembly is a common mechanism for NE assembly and expansion in all germ, embryonic, and somatic cells.

In summary, based on our present data and previous reports, we propose a simple working model to elucidate the mechanism of NE assembly and expansion in proliferating higher eukaryotic cells (Fig. 5). We propose that three membrane sources contribute to NE assembly and expansion. These membranes are the ER, the previously disassembled membrane vesicles, and the membrane of the newly assembled AL, which hosts timely ALPC/NPC assembly beyond the NE. Although the NE and NPC assembly around the separating chromatids using the existing membrane vesicles or ER components and Nup proteins during mitotic exit, the AL clearly appear to spontaneously assemble in the cytoplasm using the specialized membrane and Nup proteins. Then, the AL either bind the decondens-
ing chromatin directly and laterally fuse with the assembling NE to contribute to NE assembly or fuse with the outer nuclear membrane to transform into the NE for NE expansion and nuclear growth. Collectively, our findings not only provide insights into the mechanism of NE assembly and expansion as the NPC numbers increase, to which the AL contributes, but also provide a method to study the assembly of the NE and AL and the function of the AL during cell proliferation.

**Experimental procedures**

**Molecular cloning, protein expression, and antibodies**

Nup35, Nup93, Nup98, and Nup107 were amplified by PCR from the 293-cell cDNA library and cloned into pEGFP-C2 (Clontech, catalog no. 6083-1). The antibodies mAb414 (ab24609, Abcam), polyclonal anti-human Nup107 (A301-787A, Bethyl Laboratories), and rabbit anti-calnexin (sc-11397, Santa Cruz Biotechnology) were purchased. The rabbit anti-human LBR antibody was raised against glutathione S-transferase–tagged human LBR amino acids 1–60. All animal experiments were performed in the Laboratory Animal Center of Peking University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals according to guidelines approved by the Institutional Animal Care and Use Committee at Peking University.

**Drosophila strains and maintenance**

*Drosophila melanogaster* lines stably expressing GFP–Nup107 (Flybase Stock No. 35513) and RFP–Nup107 (Flybase...
Stock No. 35820) were obtained from the Bloomington Drosophila Stock Center. These Drosophila lines were maintained at 18 °C in vials and set up for crossing at 25 °C.

Establishment of cell lines stably expressing GFP–Nups
To establish cell lines stably expressing GFP–Nups, HeLa cells were transfected with GFP–Nups via Lipo2000 (Invitrogen), according to the manufacturer’s instructions, and selected with 10 µg/ml G418 after transfection for 72 h. Single-cell clones were individually picked and cultured in fresh medium with G418.

Immunofluorescence microscopy (IFM)
HeLa and HEK293 cells were grown on glass coverslips in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum at 37 °C in a 5% CO2 atmosphere and transfected with the appropriate vectors. IFM was carried out as described previously (38).

Time-lapse microscopy of live embryos
Syncytium blastoderm embryos were collected 90 min after eggs were laid, and late development-stage embryos were collected after 10 h. The embryos were bleached in 50% HClO4 buffer for 60 s, washed with double-distilled H2O, and immersed in halocarbon oil 700 with a coverslip. Brains from third instar larvae were dissected and mounted in explant solution as described previously (38).

TEM
Samples were fixed for 1 h with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature. After rinsing three times with 0.1 M sodium cacodylate buffer, the samples were postfixed with 1.0% OsO4 and rinsed with water. After dehydration in a graded series of acetone (10 min each), the samples were embedded in Epon 812 resin. Samples were sectioned with a diamond knife in a Leica Ultracut R cutter. After staining with uranyl acetate and lead citrate, the sections were observed under a JEM-1010 TEM, and images were captured with an AMT CCD camera.

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**ACCELERATED COMMUNICATION:** Nuclear envelope reconstitution