Live imaging system for visualizing nuclear pore complex (NPC) formation during interphase in mammalian cells

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Nuclear pore complexes (NPCs) are ‘supramolecular complexes’ on the nuclear envelope assembled from multiple copies of approximately 30 different proteins called nucleoporins (Nups) that provide aqueous channels for nucleocytoplasmic transport during interphase. Although the structural aspects of NPCs have been characterized in detail, NPC formation and its regulation, especially during interphase, are poorly understood. In this study, using the temperature-sensitive RCC1 mutant tsBN2, a baby hamster kidney 21 cell line, we found that a lack of RCC1 activity inhibited NPC formation during interphase, suggesting that RanGTP is required for NPC formation during interphase in mammalian cells. Utilizing the reversible RCC1 activity in tsBN2 cells, we established a live-cell system that allows for the inhibition or initiation of NPC formation by changes in temperature. Our system enables the examination of NPC formation during interphase in living cells. As a lack of RCC1 decreased some Nups containing unstructured phenylalanine-glycine repeats in the NPC structure, we propose that RCC1 is also involved in maintaining NPC integrity during interphase in mammalian cells.

Introduction

In eukaryotic cells, genomic DNA and the cytoplasm are physically separated by a lipid bilayer known as the nuclear envelope (NE) (Hetzer et al. 2005). This enables eukaryotic cells to regulate gene expression in a spatio-temporal manner, which may have contributed to the diversification of eukaryotes. Nuclear pore complexes (NPCs) are embedded in the NE at sites of fusion between the outer and inner nuclear membranes (ONM and INM, respectively) (Tran & Wente 2006; Antonin et al. 2008; D’Angelo & Hetzer 2008). NPCs provide aqueous channels for nucleocytoplasmic transport during interphase (Feldherr et al. 1984; Görlich & Kutay 1999; Imamoto 2000; Stewart 2007).

The NPC is a large protein complex with an estimated molecular mass of 60–125 MDa in vertebrates and 40–60 MDa in yeast. Despite the difference in their molecular masses, the fundamental architecture of NPCs is well conserved among eukaryotes. In the plane of the NE, an eightfold symmetrical perpendicular central framework, consisting of a cytoplasmic ring, spokes, and nuclear ring, form a central channel where bidirectional transport occurs (Antonin et al. 2008; D’Angelo & Hetzer 2008; Beck & Medalia 2008). Eight filaments attached to the cytoplasmic ring, called cytoplasmic fibrils, have loose ends, while eight filaments extending from the nuclear ring are attached to the distal ring forming a structure known as a nuclear basket (Antonin et al. 2008; D’Angelo & Hetzer 2008; Beck & Medalia 2008). Proteomic analyses have identified most of the components of vertebrate and yeast NPCs, and they indicate that NPCs are composed of multiple copies of approximately 30 different proteins, known as nucleoporins (Nups).
(Rout et al. 2000; Cronshaw et al. 2002). Biochemical studies have shown that Nups form various subcomplexes, which are considered to be the building blocks of NPCs (Alber et al. 2007a,b). The central channel in a NPC is filled with unstructured polypeptides containing hydrophobic phenylalanine–glycine (FG) repeats, and it serves as a selective transport barrier (Tran & Wente 2006; Antonin et al. 2008; D’Angelo & Hetzer 2008). In vertebrates, the central channel is surrounded by a framework consisting of the Nup107–160 complex, which plays an essential role in NPC architecture (Harel et al. 2003b; Walther et al. 2003a). Components of the Nup107–160 complex, including Nup107, are retained stably in the NPC with a resident time of tens of hours (Rabut et al. 2004). Vertebrate NPCs contain three integral membrane proteins (Ndc1, gp210, and Pom121), which anchor the NPC scaffold to the nuclear membrane (Antonin et al. 2005; Stavru et al. 2006; Madrid et al. 2006; Funakoshi et al. 2007; Mansfeld et al. 2006).

Although the structure of the NPC has been studied intensively, the mechanism of assembly remains largely unknown. In higher eukaryotic cells, which have open mitosis, NPC assembly occurs twice during the cell cycle: postmitosis and during interphase (Maul et al. 1972; Maeshima et al. 2006; Winey et al. 1997). Using a Xenopus egg extract system, Angelo et al. showed that NPC formation occurs during interphase by a de novo mechanism (D’Angelo et al. 2006). The small GTPase Ran is a common factor that reportedly plays a key role in NPC assembly both in yeast and vertebrates. In Xenopus eggs, the production of RanGTP was shown to be required for both postmitotic NPC assembly and NPC formation during interphase (Walther et al. 2003b; D’Angelo et al. 2006). The exact role of Ran in NPC formation during interphase is currently unknown; however, it is likely that Ran operates in NPC formation by reversing the negative effect of importin β (D’Angelo et al. 2006; Harel et al. 2003a; Ryan et al. 2007).

The RanGTPase cycle is regulated by two Ran-specific regulators: RCC1 and RanGTPase-activating protein (RanGAP) (Görlich & Kutay 1999; Nishimoto 2000; Stewart 2007). RCC1 is the guanine nucleotide exchange factor (GEF) for Ran and a chromatin-binding protein. RCC1 localizes within the nucleus, while RanGAP is a cytoplasmic protein. As a result, the concentration of RanGTP is high in the nucleus, whereas it is low in the cytoplasm (Kalab et al. 2006). The distinct RanGTP concentrations between the nucleus and cytoplasm were discovered through the study of importin-based nucleocytoplasmic transport. However, the role of Ran in NPC formation during interphase was suggested to be independent of its role in transport, because cytoplasmic RanGTP is thought to play a direct role in NPC formation (D’Angelo et al. 2006; Ryan et al. 2007).

In this study, we demonstrated that RanGTP production is required for NPC formation during interphase in mammalian cells using the temperature-sensitive RCC1 mutant tsBN2, a baby hamster kidney (BHK)21 cell line (Nishimoto et al. 1978). In tsBN2 cells, RCC1 was degraded immediately upon an upward temperature shift to a non-permissive temperature, while a downward temperature shift to a permissive temperature restored RCC1 activity (Nishitani et al. 1991; Arnaoutov et al. 2005). Immunofluorescence and photobleaching assays showed that a loss of RCC1 inhibited NPC formation during interphase. We took advantage of the reversible RCC1 activity in tsBN2 cells to establish a live-cell system that allows for the inhibition or initiation of NPC formation by a change in temperature. Furthermore, as the loss of RCC1 caused a decrease in some FG-Nups in NPCs, we propose that RCC1 is involved in the maintenance of NPC integrity during interphase. Finally, the possible roles of RCC1 in NPC maintenance during interphase are discussed.

Results

RCC1 is necessary for NPC formation during interphase in mammalian cells

To elucidate the role of RCC1 in NPC formation during interphase, we used tsBN2, a temperature-sensitive RCC1 mutant BHK21 cell line (Nishimoto et al. 1978). Because of a point mutation in RCC1 (Uchida et al. 1990), RCC1 is degraded immediately
at non-permissive temperatures (Fig. S1A in Supporting Information and Fig. 3D) (Nishitani et al. 1991; Tachibana et al. 1994). The tsBN2 cells grow normally at permissive temperatures, whereas at non-permissive temperatures, they arrest in G1 phase (Figs S1B and S3D in Supporting Information) or undergo premature chromosome condensation in S-G2 phase. These characteristics of tsBN2 cells are useful for elucidating the mechanism of formation of NPCs, which double in number during the transition from G1 to S phase.

First, we tested the effect of RCC1 depletion on NPC formation during interphase in fixed cells. To synchronize tsBN2 cells in G1 phase, cells arrested in mitosis by nocodazole were released and incubated for 2 h at a permissive temperature (32.0 °C). The cells were then incubated at either the permissive or a non-permissive temperature (39.7 °C). The total number of NPCs, nuclear volume, and NPC density were measured for each condition. The results are shown in Figure 1B-F. The data indicate that RCC1 depletion affects NPC formation during interphase.

Figure 1 Loss of RCC1 in tsBN2 cells affects NPC formation during interphase. (A) Schematic of the temperature-shift experiment. Synchronized mitotic tsBN2 cells were released and incubated at a permissive temperature (32.0 °C) for 4 h. The cells were then cultured for 18 h at the permissive or non-permissive temperature (39.7 °C). For the cells at the permissive temperature, 5 µg/ml aphidicolin was added to arrest the cells in G1/S phase (Pedrali-Noy et al. 1980). (B) RCC1 (left) and NPCs (right) in the cells grown at the permissive (top) and non-permissive temperatures (bottom) were visualized by immunostaining. The images shown are of the same intensity. The RCC1 signal in the cells cultured at the non-permissive temperature (bottom) was much lower than that in the cells cultured at the permissive temperature (top). Bar, 20 µm. (C) High-magnification images of the mAb414-labeled cells. The signal intensity of the image for the non-permissive cells (bottom) was enhanced to improve visualization. The insets show magnified images of single NPCs. Numerous cytoplasmic foci were observed in the cells cultured at the non-permissive temperature. (D–F) Scatter plots of the measured NPC density (Examined nuclei number, N = 10), nuclear volume (N = 20), and total number of NPCs per nucleus (N = 20) are shown (for details of the calculations, see Experimental procedures). Bars indicate the mean values.
non-permissive temperature (39.7 °C) for 18 h, and then fixed with formaldehyde (FA) (Fig. 1A). Aphidicolin, an inhibitor of DNA polymerase α/β, was added to control cells at the permissive temperature to inhibit further cell cycle progression (Pedrali-Noy et al. 1980). The NPCs in the cells were then visualized by immunostaining with mAb414, which recognizes the FG repeats in some Nups. Interestingly, the signal intensity of the NPCs from the cells incubated at the non-permissive temperature was significantly decreased (Fig. 1B). Moreover, many mAb414-labeled foci were detected in the cytoplasm of the cells grown at the non-permissive temperature, although a few were detected in the cells grown at the permissive temperature (Fig. 1B,C). Cytoplasmic foci were observed in 91.9% of tsBN2 cells grown at the non-permissive temperature, whereas only 3.4% of the cells grown at the permissive temperature had such foci (Fig. S2A in Supporting Information). As a control, the effect of temperature on the existence of cytoplasmic foci and NPC staining was examined in BHK21 cells (the parental line of tsBN2). A few foci were detected in the cytoplasm of BHK21 cells grown at 32.0 and 39.7 °C; however, neither temperature-specific cytoplasmic foci nor weak NPC staining was observed (Figs S2B and S3C in Supporting Information).

Next, using signal-enhanced images, the NPC density in cells grown at the permissive or non-permissive temperature was examined (Fig. 1C; for details on how the NPC density was quantified, see the Experimental procedures). By comparison, NPC density in the cells grown at the non-permissive temperature was lower than that in the cells grown at the permissive one (Fig. 1C and Inset). The average NPC density at the permissive temperature was significantly higher than that at the non-permissive temperature (P-value < 0.0001). The average measured nuclear volumes at the permissive and non-permissive temperatures were 5.373 ± 0.248 and 3.943 ± 0.487 [standard deviation (SD)] NPCs/μm², respectively (Fig. 1D). The average NPC density at the permissive temperature was significantly higher than that at the non-permissive temperature (P-value < 0.0001). The average measured nuclear volumes at the permissive and non-permissive temperatures were similar (P-value = 0.0982): 2385 ± 228 and 2204 ± 572 μm³, respectively (Fig. 1E). The estimated average total numbers of NPCs at the permissive temperature (4635 ± 296 NPCs) were thus significantly higher than that at the non-permissive temperature (3213 ± 552 NPCs) (P-value < 0.0001) (Fig. 1F).

These findings indicate that RCC1 is necessary for NPC formation during interphase in mammalian cells.

Visualization of newly formed NPCs during interphase

As described above, we manually counted the number of NPCs to determine the NPC density; however, this procedure is laborious and can lead to such errors as underestimation, both because the relatively low resolution of light microscopes can be insufficient for distinguishing adjacent NPCs and because nuclear volume increases as the cell cycle progresses. We thus attempted to establish a live-cell system for visualizing NPC formation during interphase so as to obtain additional spatio-temporal information (Fig. 2B,C). Our system utilizes fluorescently labeled Nup107, a component of NPC scaffolds, which is retained stably in NPCs and which has the longest resident time among all Nups examined thus far (>40 h) (Rabut et al. 2004; D’Angelo et al. 2009). Nup107 fused with the bright yellow fluorescent protein Venus (Nagai et al. 2002) was stably expressed in tsBN2 cells (tsBN2Venus-NUP107 cells; see Experimental procedures). On the nuclear surface in the tsBN2Venus-NUP107 cells, NPCs were observed as individual dots of Venus fluorescence. To distinguish newly formed NPCs from existing ones, the existing NPC signals from about half of the nuclear surface were photobleached using a 488-nm laser (Fig. 2A). Several hours later, newly formed dots corresponding to Venus fluorescence were detected in the bleached region (Fig. 2A). These dots likely represent new NPCs that were formed during interphase, because Nup107 is a scaffold protein and one of the most immobile NPC components (Rabut et al. 2004). It was previously shown in a myoblast fusion system that Nup107 does not exchange once incorporated into NPCs (D’Angelo et al. 2009). To analyze NPC formation, we systematically defined the Venus fluorescence signals (Fig. S3A in Supporting Information; see Experimental procedures) and examined their density every 3 h (Fig. 2B). The number of Venus dots increased in a time-dependent manner (Fig. 2D). To confirm that the formation of the fluorescent dots indeed represented NPC formation during interphase, the bleached nuclear surfaces were immunolabeled with anti-Nup153 antibodies. The number of new yellow fluorescent dots was clearly decreased compared to the Nup153 signal, which reflects the total number of NPCs on the nuclear surface (Fig. S3B in Supporting Information). Thus, our system may be used to observe NPCs formed during interphase.

We next tested the effect of RCC1 depletion on NPC formation using the live-cell system described above. Mitotically synchronized tsBN2Venus-NUP107
cells were released, incubated for 4 h at a permissive temperature, and then shifted to a non-permissive one (Fig. 2C). Photobleaching was performed at 4 h after the upward temperature shift. The bleached cells were then further incubated for 12 h at the non-permissive temperature. Almost no new NPC formation was detected in the bleached areas under these conditions (Fig. 2C). In contrast, when the cells were incubated at the permissive temperature, many fluorescent dots corresponding to NPCs were detected in the bleached areas (Fig 2A). This indicates that RCC1 is required for NPC formation during interphase in living mammalian cells.

**Manipulation of NPC formation during interphase by a temperature shift**

Using the live-cell system described above, we observed the inhibition of NPC formation at the non-permissive temperature. Thus, we examined...
whether if the temperature was shifted down to the permissive value from the non-permissive one, NPC formation would resume. To test this possibility, after their release from mitosis, synchronized tsBN2 cells were incubated at the non-permissive temperature for 12 h (Fig. 3A). Existing NPC signals on the nuclear surface of interest were then bleached, and the temperature was shifted down to the permissive value (Fig. 3A). Twelve hours after photobleaching, newly formed NPCs were detected in the bleached area as bright fluorescent dots (Fig. 3A). Time-lapse monitoring of the bleached area showed that the number of newly formed NPCs increased in a time-dependent manner (Fig. 3B,C) as seen in Fig. 2D. Thus, we concluded that NPC formation began again after the shift from a non-permissive temperature to a permissive one.

To confirm these data, the recovery rate of RCC1 activity was examined after the temperature shifts described above. We first examined the amount of active RCC1 after a downward temperature shift (Fig. 3D–F). After mitotic release, synchronized tsBN2 cells were incubated at the non-permissive temperature for 12 h, and then the temperature was

**Figure 3** Manipulation of NPC formation by a temperature shift. (A) Experimental design for the temperature-shift experiment. Mitotic tsBN2Venus-Nup107 cells were released and incubated at the non-permissive temperature for 12 h. The existing fluorescent dots on the nuclear surface of interest were bleached. Cells were incubated at the permissive temperature for an additional 12 h and then observed. NPC formation was detected after 12 h at the permissive temperature (right image). (B) The number of newly formed NPCs increased in a time-dependent manner. (C) The NPC density was examined and plotted. Error bars show the standard deviation. (D) and (E) The amount of RCC1 was examined after the temperature shift. After mitotic release, synchronized tsBN2 cells were incubated at the non-permissive temperature for 12 h. After a downward shift to the permissive temperature (0 h), the amount of RCC1 was monitored over time by immunoblotting (D) and immunofluorescence staining (E). Whole-cell lysates (equivalent to $2 \times 10^4$ cells) prepared at the indicated time points were loaded in each lane. (F) Graphical representation of (D) and (E). The amount of RCC1 was plotted as the ratio to the control value. The standard deviation is indicated by error bars.
shifted down to the permissive value (0 h). The amount of RCC1 activity was monitored over time by immunofluorescence staining and immunoblotting. Both analyses showed that RCC1 signals increased after the downward temperature shift (Fig. 3F), confirming that new NPC formation after the shift was dependent on the recovery of RCC1 in tsBN2 cells. In addition, we verified that the cell cycle arrest at G1 phase was also released upon the downward temperature shift (Fig. S3D in Supporting Information).

Our results suggest that NPC formation during interphase could be manipulated by a temperature shift in tsBN2 cells. To demonstrate the inhibition and recovery of NPC formation (Fig. S4A in Supporting Information), tsBN2Venus-NUP107 cells synchronized in G1 phase and grown at the non-permissive temperature were photobleached. The temperature was then kept at the non-permissive value for 10 h before being shifted down to the permissive value and maintained for another 12 h. Newly formed NPCs were quantified manually by counting of the NPC number (Fig. S4C in Supporting Information). At the non-permissive temperature, few NPCs were detected [Fig. S4B,C in Supporting Information; NPC density: 0.248 ± 0.227 NPCs/μm²], but after the shift down to the permissive temperature, the NPC density increased to 2.861 ± 0.412 NPCs/μm² after 12 h (Fig. S4B,C in Supporting Information). Thus, our tsBN2 system makes it possible to inhibit and restart NPC formation during interphase by a shift in temperature.

Structural integrity of NPCs is affected by RCC1 activity

In tsBN2 cells incubated at the non-permissive temperature, the signal intensity of NPCs labeled with mAb414 was decreased significantly (Fig. 1B), whereas this impairment was not observed in BHK21 cells (Fig. S3C in Supporting Information). We thus tested whether the signal intensity could be recovered after a downward shift to the permissive temperature. After 12 h at the non-permissive temperature, a significant decrease in the intensity of mAb414 staining was observed (P-value <0.0001) (Fig. 4A). However, when the temperature was shifted down to the permissive value, its intensity was almost completely recovered (Fig. 4B). The signal intensity of NPCs labeled with anti-Nup153 antibodies was also examined. Nup153 is a constituent of the NPC nuclear basket, and FG-Nups have a very high turnover rate (Rabut et al. 2004). The staining intensity of Nup153 declined at the non-permissive temperature (P-value <0.0001), but recovered upon a downward shift to the permissive temperature, as was observed using mAb414 (Fig. 4B).

To examine this finding in living cells, tsBN2 cells stably expressing Venus-Nup153 were established (tsBN2Venus-Nup153). The change in intensity of Venus-Nup153 upon the temperature shift was analyzed by live-cell imaging. Consistent with our previous results, the intensity of Nup153 decreased at the non-permissive temperature (Fig. S6B in Supporting Information), suggesting that RCC1 maintains the structural integrity of NPCs during interphase.

To further investigate NPC structure at the non-permissive temperature, we next stained tsBN2Venus-Nup107 cells with mAb414 (Fig. 5A). Although mAb414 staining at the non-permissive temperature was reduced by 35.5 ± 11.9 % (Fig. 5A,B), the intensity of Venus-Nup107 was decreased by only 11.9 ± 22.6% (Fig. 5A,B). This suggests that significant fractions of scaffold Nups were retained in the NPCs even at the non-permissive temperature. To confirm this idea, we observed NPC structure directly using cryo-scanning electron microscopy (SEM). Synchronized tsBN2 cells incubated for 12 h at the permissive and non-permissive temperatures were freeze-fractured, and the nuclear surfaces in the fractured cells were observed by SEM. Although a temperature-dependent difference in NPC density was observed (Fig. 5C, lower images), no notable difference in overall NPC structure was detected (Fig. 5C, upper images). This suggests that RCC1 depletion does not significantly affect the organization of scaffold Nups in NPCs.

Discussion

tsBN2 cells: a novel system for studying NPC formation during interphase

In this study, a novel live-cell system was established for analyzing NPC formation during interphase. In this system, we took advantage of the unique characteristics of tsBN2 cells, a temperature-sensitive mutant of RCC1(Nishimoto et al. 1978). A key feature of tsBN2 cells is the rapid degradation of RCC1 at non-permissive temperatures (Fig. S1A in Supporting Information) (Nishitani et al. 1991). Although several methods may be used to eliminate RCC1 function, the rapid depletion of RCC1 in tsBN2 cells is noteworthy. Based on this feature, we
found defects in NPC formation during interphase as a result of the loss of RCC1, not only in the fixed cells but also in living cells (Figs 1 and 2). Another notable finding is the reversibility of RCC1 activity in tsBN2 cells (Fig. 3D–F); the amount of RCC1 was restored following a shift in temperature. Under these conditions, NPC formation could be restarted. Using these unique characteristics of tsBN2 cells, we were able to establish a live-cell system for the observation of NPC formation during interphase (Fig. 3).

Figure 4 Rapid recovery of the signal intensity of mAb414 and Nup153 upon the shift from a non-permissive to a permissive temperature. (A) Immunofluorescence staining for mAb414 (top) and Nup153 (bottom) in the cells at the permissive temperature (left), non-permissive temperature (center), and following a shift to the permissive temperature from the non-permissive value (right) is shown. The images are shown using the same intensity scale for comparison. Although the intensities of the mAb414 and Nup153 signals at the non-permissive temperature were much lower than those at the permissive one, their intensities were increased significantly after incubation at the permissive temperature for 2 h. Scale bar, 10 μm. (B) Relative changes in intensity upon the temperature shift were quantified. The intensity of each NPC labeled with mAb414 (left) or Nup153 (right) in the cells (Examined nuclei number, N = 10) was measured and plotted (upper panels). Each circle in the graph indicates the mean intensity on a single nuclear surface. The bars indicate the mean values. The mean values of relative intensities of mAb414 at the permissive, non-permissive, and non-permissive-to-permissive temperatures were 1 ± 0.138 standard deviation (SD), 0.645 ± 0.119, and 0.958 ± 0.220, respectively. Those of Nup153 at the permissive, non-permissive, and non-permissive-to-permissive temperatures were 1 ± 0.121, 0.530 ± 0.126, and 0.857 ± 0.137, respectively. The same data sets are shown as bar graphs for comparison (bottom panel). SD is indicated by error bars.

Live-cell system for observing NPC formation during interphase

Compared to our understanding of postmitotic NPC assembly, our knowledge of NPC formation during interphase is limited (Antonin et al. 2008; D’Angelo & Hetzer 2008), presumably because of the lack of a practical system for analysis. The most serious obstacle to visualizing NPC formation during interphase is that NPCs already exist after NE formation, and these existing NPCs usually interfere with the observation of newly formed NPCs. To distinguish new NPCs from existing ones, we applied a photobleaching technique to the tsBN2Venus-Nup107 cell line. The signals from the existing NPCs were almost completely abrogated by photobleaching. Several hours later, we detected newly formed dots of Venus fluorescence in the bleached region (Fig. 2). If Nup107 from unlabeled NPCs can be exchanged for free Venus–Nup107, then the labeling of an NPC would not necessarily mean that it is newly formed. However, this is unlikely. First, Nup107 is a component of NPC scaffolds, which are retained stably in NPCs with a residence time exceeding 40 h (Rabut et al. 2004); moreover, it was shown that Nup107 could not be exchanged once incorporated into NPCs.
Second, if the dots of Venus fluorescence in the bleached areas are derived from an exchange for free Venus–Nup107 as in conventional fluorescence recovery after photobleaching (Houtsmuller 2005), the fluorescence recovery of bleached NPCs would occur uniformly and gradually, and NPC density in the bleached regions would be the same as in the unbleached areas. However, we observed the Venus signals as discrete dots instead of as uniform ones. The NPC density in the bleached areas was much less than that in the unbleached ones (Fig. 2D), and this result was confirmed by immunostaining (Fig. S3B in Supporting Information). We therefore concluded that the Venus fluorescent dots that appeared in the bleached areas were newly formed NPCs and that we successfully created a new live-cell system for observing newly formed NPCs during interphase, although the observed fluorescent dots might contain an intermediate or incomplete form of NPCs.

**RCC1 function in NPC formation during interphase**

How does RCC1 activity contribute to NPC formation during interphase? RCC1 is the GEF for Ran. Previously, Ryan et al. reported that the RanGTPase cycle was required for NPC assembly in yeast (Ryan et al. 2003). Angelo et al. suggested that RanGTP production is essential in *Xenopus* eggs (D’Angelo et al. 2009).
Structural maintenance of NPCs by RCC1 activity during interphase

By taking advantage of the properties of tsBN2 cells, we detected weak NPC staining with mAb414 in cells grown at the non-permissive temperature (Figs 1B, 4A and 5A). Surprisingly, the weak signal was rapidly recovered after a downward shift to the permissive temperature (Fig. 4B). Interestingly, the inhibition of Ran-dependent nucleocytoplasmic transport at the non-permissive temperature was also quickly reversed after a shift to the permissive temperature (Fig. S5D in Supporting Information). mAb414 is a monoclonal antibody that recognizes FG repeats in some Nups, including Nup153. A similar result was obtained using anti-Nup153 antibodies (Fig. 4), and also in living tsBN2Venus-Nup153 cells (Fig. S6B in Supporting Information), suggesting that a loss of RCC1 directly or indirectly affects the behavior of some FG-Nups in NPCs. In fact, at the non-permissive temperature, leakage of the Nup62-Venus signal from the cytoplasm to the nucleoplasm was detected in tsBN2Nup62-Venus cells (Fig. S6A in Supporting Information). Nup62 is one of the most abundant FG-Nups and a major constituent of the central channel, which functions as a permeable transport barrier (Hu et al. 1996). On the other hand, most Nup107, a scaffold Nup, was retained in the NPCs even at the non-permissive temperature (Fig. 5A,B). The overall structure of the NPCs as revealed by SEM was not different between the cells grown at permissive and non-permissive temperatures (Fig. 5C).

In conclusion, our results produced using a new live-cell system clearly suggest that RCC1 is involved not only in NPC formation, but also in maintaining the structural integrity of NPCs during interphase. Active transport systems regulated by RCC1 may be necessary for the maintenance of NPC structures, especially FG-Nups.

Experimental procedures

Cell culture

Baby hamster kidney 21 and tsBN2 cells were generously provided by Dr T. Nishimoto. The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a humidified incubator under 5% CO₂. The BHK21 cells were maintained at 37 °C, whereas the tsBN2 cells were cultured at 32.0 (permissive temperature) or 39.7 °C (non-permissive temperature).

To synchronize the BHK21 and tsBN2 cells in metaphase, the cells were incubated with 0.1 μg/ml nocodazole (Sigma)
for 4 h. Mitotic cells were collected by shaking the culture dishes, washed three times with phosphate-buffered saline (PBS), and seeded again onto poly-ℓ-lysine-coated coverslips or glass-bottom dishes (Iwaki, Tokyo). To avoid forming binucleate cells after cytokinesis, the released tsBN2 cells were pre-incubated for 4 h at 32.0 °C to ensure the completion of cytokinesis. After incubation, the cells were used in our temperature-shift experiments.

Cell cycle profiles of the synchronized cells were produced using a laser-scanning cytometer (LSC2; Olympus, Tokyo, Japan) to quantify the DNA after treatment of the fixed cells with 100 μg/ml propidium iodide and 1 mg/ml RNase (both Sigma) in PBS at 37.0 °C for 30 min.

**Immunofluorescence**

Cells grown on poly-ℓ-lysine-coated coverslips were rinsed once with PBS and fixed with 2% FA for 15 min. Immunofluorescence was carried out at room temperature as described previously ((Maeshima & Laemmli 2003; Maeshima et al. 2006)), with minor modifications. The primary antibodies and dilutions used in our experiments were as follows: goat polyclonal anti-RCC1 antibodies (Santa Cruz Biotechnology), 1 : 100; the mouse monoclonal antibody mAb414 (abcam), 1 : 200; and a rat monoclonal anti-Nup153 antibody (prepared as described previously in (Fukuhara et al. 2006)), 1 : 20. The secondary antibodies used were Alexa 594 donkey anti-goat (Molecular Probes), Alexa 488 goat anti-mouse, and Alexa 594 goat anti-rat antibodies at a 1 : 1000 dilution.

**NPC density, nuclear volume, and the total number of NPCs per nucleus**

NPCs on the nuclear surfaces were visualized by immunolabeling using mAb414. Images of the nuclear surfaces adjacent to the coverslips were recorded as Z-stacks using a DeltaVision restoration microscope (Applied Precision) with a 100× objective lens (Olympus). Maximum nuclear surface images were recorded before and after photobleaching (Fig. 2A–C).

Quantification of the newly formed NPCs, the mean fluorescence intensity in an unbleached area was measured and used as a threshold value to define the newly formed fluorescent dots. In a bleached area (Fig. S3A in Supporting Information, left image), regions whose intensity exceeded the threshold value were labeled (Fig. S3A in Supporting Information, center image). The number of labeled regions, which were regarded as newly formed NPCs, was counted manually. With this procedure, the NPC density in the bleached areas was obtained (Figs 2D and 3C; Fig. S4C in Supporting Information).

**Plasmid construction**

P_{EF-1α}-Venus-hNUP107 was created as follows. The coding region of Venus was amplified by PCR from Venus cDNA [a gift from Dr A. Miyawaki (RIKEN, Wako, Japan)]. The amplified fragment was then subcloned into pEGFP-C3 (Clontech) to replace the EGFP region (Venus-C3 vector). The coding region of human NUP107 was amplified from its cDNA (a gift from Dr V. Doye [Curie Institute, Paris, France]) and inserted into Venus-hNUP107. For moderate expression, the cytomegalovirus (CMV) promoter in Venus-hNUP107 was replaced with the human EF1α promoter to produce P_{EF-1α}-Venus-hNUP107, P_{EF-1α}-Venus-NUP153 and P_{EF-1α}-NUP62-Venus were constructed using the multistite Gateway system (Invitrogen) as described previously (Yahata et al. 2007). To stably establish tsBN2 cells expressing Venus-hNUP107, P_{EF-1α}-Venus-hNUP107 (100 ng) was transfected into tsBN2 cells using an Effecten Transfection Reagent Kit (Qiagen), and the transformants were selected using 1.5 mg/ml G418 (Gibco). The localization of Venus-Nup107 in the tsBN2Venus-Nup153 cells was confirmed by fluorescence microscopy. The Flp-In System (Invitrogen) was used to create the tsBN2Venus-Nup153 and tsBN2Nup62-Venus lines. A pFRT-bla construct was transfected into tsBN2 cells using an Effecten Transfection Reagent Kit (Qiagen), and the transformants were selected using 5 μg/ml blasticidin (Invitrogen) and used for the isolation of stable transformants. The isolation procedure was described previously in (Yahata et al. 2007).

**Live-cell imaging and photobleaching**

For live-cell imaging and our photobleaching experiments, tsBN2 cells on glass-bottom dishes were set in a live-cell chamber (MI-IBC; Olympus) on the stage of a DeltaVision microscope. Nuclear surface images were recorded before and after photobleaching with a 60× objective lens (Olympus). Photobleaching was performed in the region of interest using a quantitative laser module (50 mW, 488 nm solid-state laser with a ten spacing pattern) on a DeltaVision microscope (Tahara et al. 2008). Images were acquired several hours after photobleaching (Fig. 2A–C).

For quantification of the newly formed NPCs, the mean fluorescence intensity in an unbleached area was measured and used as a threshold value to define the newly formed fluorescent dots. In a bleached area (Fig. S3A in Supporting Information, left image), regions whose intensity exceeded the threshold value were labeled (Fig. S3A in Supporting Information, center image). The number of labeled regions, which were regarded as newly formed NPCs, was counted manually. With this procedure, the NPC density in the bleached areas was obtained (Figs 2D and 3C; Fig. S4C in Supporting Information).

**Quantification of RCC1 recovery after a temperature shift**

For immunofluorescence, after incubation at 39.7 °C (non-permissive temperature) for 12 h, tsBN2 cells were subjected to the permissive temperature (32.0 °C). Cells were fixed at
the indicated time points in Fig. 3F, and immunolabeled with an anti-RCC1 antibody. The tsBN2 cells were also incubated at 32 °C with 5 μg/ml aphidicolin as a control. The mean intensities of the nuclear RCC1 signals in a total of ten cells were determined, and their averaged values were compared with those of the control cells. For immunoblot analysis, tsBN2 cells were manipulated as described above. Whole-cell lysates were prepared every 4 h after the downward temperature shift (Fig. 3D). Whole-cell lysates of unsynchronized tsBN2 cells were used as a control. Signal quantification was done using LAS3000 (Fujifilm, Tokyo, Japan).

Analysis of nuclear import and export

Nucleocytoplasmic transport in tsBN2 cells was examined based on the diffusible sizes of EGFP-M9 and EGFP-Rev-NES. EGFP-M9 and EGFP-Rev-NES (Yokoya et al. 1999) were transiently expressed in tsBN2 cells as described above. Two days after transfection, the incubation temperature was shifted up to 39.7 °C (non-permissive temperature) and maintained for 10 h. After fixation with 2% FA, transport activity was evaluated by EGFP localization in the cells (Fig. S5A,B in Supporting Information). In our live-cell experiments, after the incubation, temperature was shifted to the non-permissive non-permissive temperature (permissive temperature), EGFP signals in the cells were monitored every hour (Fig. S5C in Supporting Information). When the temperature was shifted from 39.7 (non-permissive temperature) to 32.0 °C (permissive temperature), localization of the EGFP signals in the cells was monitored every 10 min (Fig. S5D in Supporting Information).

Cryo-SEM

Synchronized mitotic tsBN2 cells were seeded onto poly-L-lysine-coated metal specimen holders and cultured for 12 h at the permissive or non-permissive temperature. The cells were then rapidly frozen in liquid propane (−184 °C) and fractured in a cryo-transfer system (Alto-2500; Oxford, Oxon, UK) at −120 °C. The fractured cells were then coated with chromium and observed using a field emission scanning electron microscope (LE01530; LEO, Oberkochen, Germany).

Acknowledgements

We are grateful to Ms S. Hihara for providing technical assistance. We thank Drs T. Nishimoto, Y. Yoneda, K. Wilson, A. Miyawaki, T. Nagai, and V. Doye for their generous gifts of materials. We also thank the members of the Cellular Dynamics Lab at RIKEN and Dr Nishijima at NIG for many helpful discussions. This work was supported by a grant-in-aid from MEXT, Special Project Funding for Basic Science (Bioarchitect Project) from RIKEN, and RIKEN R&D (President’s Discretionary Fund).

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**Supporting Information/Supplementary material**

The following Supporting Information can be found in the online version of the article:

**Figure S1** (A) RCC1 in tsBN2 cells was degraded immediately after incubation at a non-permissive (39.7 °C) temperature. (B) The tsBN2 cells incubated at the non-permissive temperature were arrested in G1 phase. (C) Highmagnification images of the nuclear surfaces in tsBN2 cells incubated at the permissive (i) or non-permissive temperature (ii).

**Figure S2** (A) The number of cells with mAb414-labeled cytoplasmic foci was determined. (B) Temperature-specific cytoplasmic foci were not detected in the parental line BHK21, both at 32.0 (left image) and 39.7 °C (right image). (C) Cycloheximide, a translation inhibitor, suppressed the formation of cytoplasmic foci at the non-permissive temperature.

**Figure S3** (A) Quantification of newly formed NPCs during interphase, based on the formation of Venus dots in bleached areas. (B) Verification that the new Venus dots in the bleached region were newly formed NPCs. (C) The effect of temperature on the NPC signals was examined in BHK21 cells (the parental line of tsBN2). (D) The cell cycle resumed after the shift from the non-permissive to the permissive temperature.

**Figure S4** NPC formation resumed just after the shift from a non-permissive to a permissive temperature.

**Figure S5** (A) and (B) Nucleocytoplasmic transport (import and export) in tsBN2 cells incubated at a permissive or non-permissive temperature was examined using transiently expressed EGFP-M9 or EGFP-Rev-NES. (C) Nucleocytoplasmic transport in tsBN2 cells upon the temperature shift was monitored by time-lapse imaging. (D) Recovery of nucleocytoplasmic transport following a downward temperature shift.

**Figure S6** (A) Nuclear leakage of Nup62 at the non-permissive temperature. tsBN2Nup62-Venus cells were monitored at the permissive temperature and then at the non-permissive temperature by time-lapse imaging. (B) Attenuation of Venus-Nup153 signals in tsBN2 Venus-Nup153 cells upon the shift to a non-permissive temperature.

Additional Supporting Information may be found in the online version of this article.

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