Changes in Nucleoporin Domain Topology in Response to Chemical Effectors

Sara M. Paulillo¹, Maureen A. Powers², Katharine S. Ullman³ and Birthe Fahrenkrog¹*

¹M.E. Müller Institute for Structural Biology, Biozentrum University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland
²Department of Cell Biology Emory University School of Medicine, 615 Michael Street Atlanta, GA 30322, USA
³Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah Salt Lake City, Utah, USA

Nucleoporins represent the molecular building blocks of nuclear pore complexes (NPCs), which mediate facilitated macromolecular trafficking between the cytoplasm and nucleus of eukaryotic cells. Phenylalanine-glycine (FG) repeat motifs are found in about one-third of the nucleoporins, and they provide major binding or docking sites for soluble transport receptors. We have shown recently that localization of the FG-repeat domains of vertebrate nucleoporins Nup153 and Nup214 within the NPC is influenced by its transport state. To test whether chemical effectors, such as calcium and ATP, influence the localization of the FG-repeat domains of Nup153 and Nup214 within the NPC, we performed immuno-electron microscopy of Xenopus oocyte nuclei using domain-specific antibodies against Nup153 and Nup214, respectively. Ca²⁺ and ATP are known to induce conformational changes in the NPC architecture, especially at the cytoplasmic face, but also at the nuclear basket of the NPC. We have found concentrations of calcium in the micromolar range or 1 mM ATP in the surrounding buffer leaves the spatial distribution of the FG-repeat of Nup153 and Nup214 largely unchanged. In contrast, ATP depletion, calcium store depletion by EGTA or thapsigargin, and high concentrations of divalent cation (i.e. 2 mM Ca²⁺ and 2 mM Mg²⁺) constrain the distribution of the FG-repeats of Nup153 and Nup214. Our data suggest that the location of the FG-repeat domains of Nup153 and Nup214 is sensitive to chemical changes within the near-field environment of the NPC.

Introduction

Nuclear pore complexes (NPCs) are supramolecular assemblies embedded in the double membrane of the nuclear envelope (NE) that mediate diffusion of small molecules and ions as well as facilitated, signal-dependent transport of proteins and RNPs between the nucleus and cytoplasm of eukaryotic cells.¹,² The ∼120 MDa vertebrate NPC³ is composed of a set of ∼30 different proteins,⁴ known as nucleoporins (Nups). According to electron microscopy studies, mainly in Xenopus oocytes, the NPC is composed of a central framework that is continuous with a cytoplasmic and a nuclear ring moiety.⁵ The cytoplasmic ring moiety is decorated by eight, short, kinky filaments, whereas a nuclear basket, an assembly of eight filaments that join into a distal ring, tops the nuclear ring moiety.¹⁰ The central framework exhibits 8-fold rotational symmetry and it encloses the central pore of the NPC, which mediates all trafficking between the nucleus and cytoplasm. The central pore has a length of about 90 nm and it is narrowest in the midplane of the NE with a diameter of about 45–50 nm.⁸ The physical and functional diameters of the central pore coincide as cargo up to 39 nm is able to pass through the NPC.¹²

The NE is continuous with the endoplasmic reticulum (ER) and, as such, the NE lumen acts, together with that of the ER, as a calcium (Ca²⁺) store. Depletion of the luminal Ca²⁺ stores inhibits diffusion of 10 kDa dextrans in both cultured

Abbreviations used: NPC, nuclear pore complex; NE, nuclear envelope; Nup, nucleoporin; AFM, atomic force microscopy.

E-mail address of the corresponding author:
birthe.fahrenkrog@unibas.ch

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mammalian cells and in *Xenopus* oocytes. The effect of Ca\(^{2+}\) store depletion on facilitated nuclear import is controversial: inhibition has been observed in some studies but not in others. Atomic force microscopy (AFM) studies further showed that depletion of nuclear Ca\(^{2+}\) stores or variations in extranuclear calcium concentrations led to conformational changes within the NPC. Such changes included the appearance of a central plug on the cytoplasmic and nuclear face of the NPC, or alterations in the arrangement of the nuclear basket by ~20–30 nm. ATP can influence NPC conformation as well, and in this context AFM studies revealed that addition of ATP causes dramatic conformational changes on the cytoplasmic surface of the NPC. A direct link between changes in NPC conformation and nuclear transport has not been established, although it has been speculated that conformational changes of the NPC could alter the accessibility of FG (phenylalanine-glycine)-repeat sites within the NPC, which, in turn, should affect nuclear transport.

Facilitated nuclear transport requires the simultaneous interaction of soluble transport receptors with the NPC and with transport cargo. The interaction of the receptors with the NPC is mediated mainly by a subset of nucleoporins that harbor FG-repeat domains. Repeat domains are composed of hydrophobic FG patches that are spaced by hydrophilic linkers of variable length and sequence. Studies in yeast revealed that FG-repeat domains have little secondary structure, i.e. they are natively unfolded. These findings are supported by X-ray crystallography data, as well as by immuno-EM studies. AFM studies further document that the FG-repeat domain of Nup153 resembles an unfolded polypeptide chain with a length of about 200 nm and a width of about 0.4 nm.

We have recently mapped the domain topology of two vertebrate nucleoporins, Nup153 and Nup214, within the NPC of *Xenopus* oocytes. The non-FG-repeat domains of both nucleoporins have a tightly constrained distribution, whereas, in both cases, the FG-repeat domains are positioned flexibly within the NPC. Moreover, the spatial distribution of the FG-repeat domains of Nup153 and Nup214 changes in a transport-dependent manner, suggesting that the location of FG-repeat domains of Nup153 and Nup214 correlates with cargo/receptor interactions at the NPC. To test whether the flexibility of the FG-repeat domains of Nup153 and Nup214 is altered under conditions that lead to changes in NPC conformation, we isolated nuclei from *Xenopus* oocytes, incubated them in buffer solutions that vary in the concentration of either Ca\(^{2+}\) or ATP, and mapped the domain topology of Nup153 and Nup214 by immunogold-EM using domain-specific antibodies. Our data show that concentrations of high salt in buffer solutions, the release of luminal Ca\(^{2+}\) stores by thapsigargin, and ATP-depletion lead to distinct changes in the spatial distribution of the FG-repeat domains of Nup153 and Nup214, whereas a moderate increase in external levels of calcium or ATP has no significant influence on their location. These findings suggest that the variations in FG-repeat accessibility are due to physical changes in response to alterations in the chemical near-field environment of the NPC.

**Results**

**The influence of increasing concentrations of Ca\(^{2+}\) on the location of FG-repeat domains**

In resting cells, the concentrations of free calcium in the cytoplasm and in the nucleus appear to be approximately equivalent, and in the nanomolar range. Addition of micromolar amounts of calcium to the buffer surrounding isolated nuclei leads to an increase in the concentration of calcium in the nucleus due to diffusion of Ca\(^{2+}\) through the NPC. AFM studies in *Xenopus* oocyte nuclei have shown that addition of Ca\(^{2+}\) (to a final concentration of 100 μM) to Ca\(^{2+}\)-free buffer leads to an opening of the distal ring of the nuclear basket by 20–30 nm without affecting the height of the nuclear basket. The FG-repeat domain of Nup153 is tethered to the distal ring of the nuclear basket via the zinc-finger domain of Nup153, and can extend from there to the cytoplasmic face of the NPC, whereas the FG-repeat domain of Nup214 can extend as far as the nuclear basket from its anchoring site near or at the cytoplasmic ring moiety of the NPC.

To analyze whether an increase in Ca\(^{2+}\) and the related conformational changes of the NPC at the level of the nuclear basket affect the epitope exposure of the flexible FG-repeat domains of Nup153 and Nup214 and the stationary zinc-finger domain of Nup153 (Figure 1(a)), we isolated nuclei from *Xenopus* oocytes, incubated the nuclei in low-salt buffer (LSB) containing 100 μM Ca\(^{2+}\) for 10 min and then fixed and labeled the nuclei with antibodies against the FG-repeat domains of Nup153 (anti-Nup153-C2) and Nup214 (anti-Nup214-D), respectively. At equilibrium (i.e. in LSB without addition of calcium), the FG-repeat domain of Nup153 localizes predominantly to the nuclear side of the NPC (89%), but about 11% of the FG-repeats can be detected at the cytoplasmic face (Figure 1(b)). Incubating the nuclei with 100 μM Ca\(^{2+}\) did not change the nuclear to cytoplasmic ratio of the distribution of the FG-repeats of Nup153 (85% nuclear versus 15% cytoplasmic; Figure 1(c)). However, a slight shift from the distal ring of the nuclear basket towards the nuclear ring moiety could be observed.

In the case of Nup214, at equilibrium state ~14% of the FG-repeat domains of Nup214 localize to the nuclear face of the NPC, whereas the remaining ~86% reside on the cytoplasmic face of the NPC (Figure 1(d)). In the presence of 100 μM Ca\(^{2+}\), this overall distribution of the Nup214 FG-repeat domains is similar (21% nuclear versus 79% cytoplasmic; Figure 1(e)). Similarly, the presence of
Figure 1. Domain organization and localization of Nup153 and Nup214. (a) A representation of the domain structure of *Xenopus* Nup153 and human Nup214. Antibodies were raised against Nup153 amino acid residues 655–926 (anti-Nup153-Z), 1375–1602 (anti-Nup153-C2) and Nup214 amino acid residues 1684–2091 (anti-Nup214-D). Schematic representation and quantification of the steady-state localization of the FG-repeat domains of (b) Nup153 and (d) Nup214 as well as (f) the zinc-finger domain of Nup153 in isolated *Xenopus* nuclei. Intact isolated nuclei were collected in low-salt buffer (LSB) and immunolabeled with the anti-Nup153-C2 antibody conjugated directly to 8 nm colloidal gold, anti-Nup214-D antibody and a secondary anti-rabbit IgG antibody conjugated to 10 nm colloidal gold, and anti-Nup153-Z antibody conjugated directly to 8 nm colloidal gold and prepared for EM by Epon embedding and thin-sectioning. The center of each location cloud represents the mean distance from the central plane of the NE, and the radii are defined by the standard deviation of the vertical distances and the width of the NPC. Full color, highest probability; subdued color, less probability. The following numbers of gold particles were scored for the individual experiments: 140 (anti-Nup153-C2, steady state), 259 (anti-Nup214-D, steady state), and 89 (anti-Nup153-Z, steady state). Incubating the isolated *Xenopus* nuclei in LSB containing 100 μM Ca^{2+} before immunolabeling with (c) the anti-Nup153-C2 antibody, (e) the anti-Nup214-D, or (g) the anti-Nup153-Z antibody does not affect the localization of the corresponding domains significantly. Shown are stretches along the gold-labeled NE reflecting the (left-hand panels) typical labeling pattern for the individual antibody. Quantification of the labeling distribution is shown in the middle panels. Right-hand panels show representations of the epitope distribution revealed by the corresponding domain-specific antibodies in the form of location clouds. The following numbers of gold particles were scored for the individual experiments: 83 (anti-Nup153-C2, 100 μM Ca^{2+}), 90 (anti-Nup214-D, 100 μM Ca^{2+}), and 68 (anti-Nup153-Z, 100 μM Ca^{2+}). c, cytoplasm; n, nuclear. The scale bars represent 100 nm.
100 μM Ca²⁺ does not change the position of the zinc-finger domain of Nup153 within the NPC substantially (Figure 1(f) and (g)), although some cytoplasmic labeling was observed in the presence of 100 μM Ca²⁺. Thus, we conclude that conformational changes at the level of the nuclear basket, such as those reported in response to 100 μM Ca²⁺, do not necessarily correlate with changes in the spatial distribution of the FG-repeat domains of Nup153 and Nup214.

To mimic conditions of a localized Ca²⁺ flux from the ER, where the concentration of Ca²⁺ can transiently reach the millimolar range, we studied the effect of 2 mM Ca²⁺ on the distribution of Nup153 and Nup214 FG-repeat domains within the NPC. As shown in Figure 2(a), the presence of 2 mM Ca²⁺ led to an almost exclusively nuclear localization of the FG-repeat domain of Nup153 (98%), predominantly at the distal ring of the nuclear basket (86%). Under the same conditions, the FG-repeat domains of Nup214 also shift towards the nuclear face of the NPC (50% versus 14% at equilibrium state; Figure 2(b)), whereas the location of the zinc-finger domain of Nup153 is unchanged (Figure 2(c)). However, these changes in the distribution of the FG-repeat domains are not Ca²⁺-
specific, as 2 mM Mg\(^{2+}\) caused a similar, or even more extensive, shift of the FG-repeat domains of Nup153 and Nup214 towards the nuclear face of the NPC (Figure 3(a) and (b)).

To test if the calcium-induced redistribution of the Nup153 and Nup214 FG-repeat domains is reversed following calcium chelation, isolated nuclei from *Xenopus* oocytes were first incubated in buffer containing 2 mM Ca\(^{2+}\) and next in buffer containing 2 mM EGTA (for 10 min each) before labeling with the domain-specific antibodies. As shown in Figure 4(a) and (b), calcium-induced changes in the distribution of the Nup153 and Nup214 FG-repeat domains are only partially reversible by application of 2 mM EGTA. To confirm that calcium was quenched adequately by EGTA under these conditions, we next incubated nuclei from *Xenopus* oocytes for 10 min in buffer containing 2 mM Ca\(^{2+}\) and 2 mM EGTA, and found that in the presence of 2 mM EGTA, the distribution of the FG-repeat domains of Nup153 and Nup214 is similar to the equilibrium state (Supplementary Data Figure S1(a) and (b)). Again, no effect on the location of the zinc-finger domain of Nup153 was observed (Supplementary Data Figure S1(c)).

A rise in the concentration of cytoplasmic calcium above 300 nM was found to decrease both ATP-dependent nuclear transport and passive diffusion in intact cells,\(^{29}\) whereas no inhibition of nuclear import in response to increased calcium concentrations could be observed in permeabilized cells.\(^{13}\) To exclude the possibility that the redistribution of the FG-repeat domains of Nup153 and Nup214 was due to inhibition of nuclear transport in the isolated nuclei, we followed nuclear import of nucleoplasmin conjugated directly to 8 nm colloidal gold after incubation of the nuclei with either 100 μM Ca\(^{2+}\) or 2 mM Ca\(^{2+}\). We found that under these conditions the import of nucleoplasmin in the isolated *Xenopus* nuclei was not inhibited (Supplementary Data Figure S2(a)). Similarly, no inhibition of import of GFP-labeled nucleoplasmin in digitonin-permeabilized HeLa cells (Supplementary Data Figure S2(b)) or export of mRNA from *Xenopus* nuclei (data not shown) were observed.

Taken together, a high concentration of divalent cations within the local nuclear environment influences the localization of the FG-repeat domains of nucleoporins Nup153 and Nup214, but this does not interfere significantly with nuclear transport.

![Figure 3](image)

**Figure 3.** Mg\(^{2+}\)-dependent domain topology of Nup153 and Nup214. Intact isolated *Xenopus* oocyte nuclei were incubated in buffer containing 2 mM Mg\(^{2+}\) before labeling with (a) an antibody against the FG-repeat domain of Nup153 (anti-Nup153-C2) and (b) an antibody against the FG-repeat domain of Nup214. Shown are stretches along the NE with the typical labeling pattern (second panels) as well as the quantitative analysis of the gold particles associated with the NPC (third panels) and a summary of the gold particle distribution within the NPC. The following numbers of gold particles were scored for the individual experiments: 97 (anti-Nup153-C2, 2 mM Mg\(^{2+}\)), and 174 (anti-Nup214-D). Steady-state values are as shown in Figure 1 and are included here for reference (first panels). c, cytoplasm; n, nuclear. The scale bars represent 100 nm.
Depletion of nuclear calcium stores influences the distribution of FG-repeat domains

Since the calcium levels that influenced domain distribution corresponded to levels obtained by calcium flux, we next aimed to map the distribution of the FG-repeat domains of Nup153 and Nup214 under various conditions known to modify lumenal stores of Ca\(^{2+}\), which can be depleted, for example, by the calcium chelator EGTA or by the Ca\(^{2+}\)-uptake pump inhibitor, thapsigargin.\(^{15,32}\) To analyze the effect of calcium release from lumenal stores of calcium on the localization of the FG-repeat domains of Nup153 and Nup214 and the zinc-finger domain of Nup153, we incubated isolated nuclei from Xenopus oocytes in buffer containing 10 mM EGTA for 10 min or in buffer containing 1 \(\mu\)M thapsigargin for 30 min before labeling with the corresponding antibodies (third panels) and a summary of the labeling pattern (fourth panels). The following numbers of gold particles were scored for the individual experiments: 68 (anti-Nup153-C2, 2 mM Ca\(^{2+}\)/2 mM EGTA seq.), and 168 (anti-Nup214-D, 2 mM Ca\(^{2+}\)/2 mM EGTA seq.). Steady-state values are as shown in Figure 1 and are included here for reference (first panels). c, cytoplasm; n, nuclear. The scale bars represent 100 nm.

Increased concentrations of ATP and nucleoporin domain topology

Nuclear transport is energy-dependent and, as such, ATP-dependent, and high concentrations of
ATP are associated with structural changes at the cytoplasmic face of the NPC. 21 To test whether increased concentrations of ATP affect the domain topology of Nup153 and Nup214, we incubated isolated nuclei from Xenopus oocytes in buffer containing 1 mM ATP. The nuclei were incubated for various lengths of time and then labeled with anti-Nup153 or anti-Nup214 antibodies, respectively. We found that increasing the concentration of ATP by 1 mM does not affect the accessibility of the FG-repeat domain of Nup153 after incubation for various length of time significantly, although a slight reduction in the labeling of the cytoplasmic face could be observed (Supplementary Data Figure S4(a)). Similarly, we did not detect significant alterations in the distribution of the FG-repeat domains of Nup214 or the zinc-finger domain of Nup153 in the presence of high concentrations of ATP (Supplementary Data Figure S4(b) and (c)).

ATP-depletion limits the flexibility of the FG-repeat domains of Nup153 and Nup214

We then asked whether ATP-depletion affects the domain topology of Nup153 and Nup214. We incubated Xenopus oocyte nuclei in buffer containing 2 units/ml of apyrase for 30 min to deplete ATP33,34 before labeling with our domain-specific antibodies. After treatment with apyrase, the anti-Nup153-C2 antibody predominantly decorates the distal ring of the nuclear basket (77% of the gold particles), whereas the cytoplasmic labeling decreases (6% versus 11% at steady state; Figure 6(a)). Similarly, ATP-depletion by apyrase results in an increase of the nuclear localization of the FG-repeat domains of Nup214 (45% versus 14%; Figure 6(b)), whereas the location of the zinc-finger domain of Nup153 remains unaffected by ATP-depletion (data not shown). The data suggest that ATP-depletion affects the distribution of FG-repeat domains, particularly of Nup214.

Discussion

Domain-specific antibodies against the nucleoporins Nup153 and Nup214 have allowed mapping of the domain topology of these two nucleoporins within the NPC and have revealed that both harbor flexible FG-repeat domains.26,27 Moreover, these domain-specific antibodies have been shown to be a powerful tool to explore possible changes in nucleoporin domain topology in a nuclear transport-dependent manner.27 In the present study, we have used domain-specific antibodies against Nup153 and Nup214 to investigate nucleoporin domain topology with respect to NPC conformation. The NPC conformation and topology have been shown by AFM and scanning EM to be
affected by chemical effectors, such as Ca$^{2+}$ and ATP.\textsuperscript{16,17,20,32,35} Our data suggest that gross structural changes at the level of the NPC as observed by AFM and scanning EM do not coincide with changes in the domain topology of the nucleoporins Nup153 and Nup214. However, the flexibility of the FG-repeat domains of both nucleoporins is affected by Ca$^{2+}$ as well as ATP.

### Calcium and nucleoporin domain topology

In isolated Xenopus oocyte nuclei, AFM studies have documented an opening and closing of the distal ring of the NPC nuclear basket by 20–30 nm in the presence or in the absence of 100 μM Ca$^{2+}$ in the surrounding buffer, respectively.\textsuperscript{20} Moreover, cryo-EM studies on isolated Xenopus nuclei in the presence or in the absence of 100 μM Ca$^{2+}$ revealed significant differences in the radial mass density profiles of the central framework (Figure 7). In calcium-free buffer, i.e. low-salt buffer, the central framework appears more massive (Figure 7(a)) as compared to the central framework of NPCs treated with 100 μM Ca$^{2+}$ (Figure 7(b)). Additionally, NPCs in the absence of Ca$^{2+}$ yield more mass in the central pore, which might reflect cargo in transit, than NPCs that have been treated with calcium.

In the present immuno-EM study, we observed neither an effect by 100 μM Ca$^{2+}$ on the topology and flexibility of the FG-repeat domains of Nup153 and Nup214 nor the location of the zinc-finger domain of Nup153, which resides at the distal ring of the nuclear basket (see Figure 1). Nup153 and Nup214 are peripheral nucleoporins that are anchored to the nuclear and cytoplasmic ring moiety, respectively, and do not contribute to the architecture of the central framework of the NPC.\textsuperscript{36,37} Our data suggest, therefore, that conformational changes on the level of the NPCs central framework do not correlate with changes in the topology of the FG-
repeat domains of peripheral nucleoporins, indicating that their mobile character is not constrained by the conformation of the central framework.

The presence of higher concentrations of calcium, i.e. 2 mM Ca\(^{2+}\), caused the redistribution of the FG-repeat domains of Nup153 and Nup214 within the NPC, whereas the location of the stationary zinc-finger domain of Nup153 was unaffected (see Figure 2). This effect on the spatial distribution of the FG-repeat domains, however, appears to be due to charge effects, since 2 mM Mg\(^{2+}\) had a similar effect on the location of the FG-repeat domains of Nup153 and Nup214 (Figure 3). FG-repeat domains are natively unfolded \(^{23,24,28}\) and, as such, they are characterized by a large net charge and hence sensitivity to temperature, pH, and ionic strength.\(^{38}\) Under physiological temperature, pH and ionic strength, natively unfolded proteins are unstructured mainly because of electrostatic repulsion between non-compensated charges.\(^{38}\) This electrostatic repulsion, which, in turn, coincides with a partial folding of the protein, can be reduced by oppositely charged ions, as they are introduced by a shift in the pH or upon addition of salts.\(^{38}\) High concentrations of divalent cations, such as 2 mM Ca\(^{2+}\) or 2 mM Mg\(^{2+}\), in the near-field of the FG-repeat domains therefore most likely cause their collapse, i.e. a partial folding that constrains their mobility.

Depletion of nuclear calcium stores by thapsigargin constrained the flexibility of the FG-repeat domains of both Nup153 and Nup214 (Figure 5(a) and (b)), whereas depletion of the calcium store by EGTA had a milder effect on the FG-repeats (Supplementary Data Figure S3). Thapsigargin blocks the sarcoplasmic/endoplasmic reticulum calcium ATPases and, therefore, is predicted to deplete lumenal calcium stores in the NE and the calcium ATPases and, therefore, is predicted to blocks the sarcoplasmic/endoplasmic reticulum (Supplementary Data Figure S3). Thapsigargin causes a virtually irreversible depletion of Nup153 and Nup214 (Figure 3). FG-repeat domains are natively unfolded \(^{23,24,28}\) and, as such, they are characterized by a large net charge and hence sensitivity to temperature, pH, and ionic strength.\(^{38}\) Under physiological temperature, pH and ionic strength, natively unfolded proteins are unstructured mainly because of electrostatic repulsion between non-compensated charges.\(^{38}\) This electrostatic repulsion, which, in turn, coincides with a partial folding of the protein, can be reduced by oppositely charged ions, as they are introduced by a shift in the pH or upon addition of salts.\(^{38}\) High concentrations of divalent cations, such as 2 mM Ca\(^{2+}\) or 2 mM Mg\(^{2+}\), in the near-field of the FG-repeat domains therefore most likely cause their collapse, i.e. a partial folding that constrains their mobility.

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**ATP and nucleoporin domain topology**

AFM studies have previously revealed that addition of ATP (to 1 mM final concentration) to isolated Xenopus nuclei induces conformational changes on the cytoplasmic face of the NPC.\(^{21}\) The data presented here show that these ATP-dependent conformational changes do not correlate with changes within the topology of the FG-repeat domains of Nup153 and Nup214 (Supplementary Data Figure S4), which most likely is due to the high flexibility of these nucleoporin domains. In contrast, ATP-depletion constrains the flexibility of the FG-repeat domains of both Nup153 and Nup214, indicating that FG-repeat flexibility is influenced by an active process that requires energy. Similar to our previous findings,\(^{22}\) limited FG-repeat flexibility under these conditions might be due to an arrest in nuclear transport, since nuclear transport is known to be ATP-dependent.\(^{34}\)

Taken together, our findings suggest that the spatial distribution of flexible FG-repeat domains is not influenced significantly by gross conformational changes in the central framework or the central pore of the NPC, such as the appearance and movement of a central plug, mass redistribution within the central framework, or the opening and closing of the distal ring of the nuclear basket. However, increased concentrations of divalent cation as well as ATP-depletion, coincide with a constrained flexibility of the FG-repeat domains of Nup153 and Nup214, further strengthening the notion that FG-repeat domains are natively unfolded, and that nucleoporin topology within the NPC architecture is influenced by energy-dependent processes.

These data underscore the multi-layered nature of NPCs dynamics and plasticity. Whereas gross changes in NPC architecture have been documented by AFM and scanning electron microscopy studies,\(^{16,17,20,32,35}\) dynamics in nucleoporin residence at the NPC have been revealed by real-time imaging,\(^{42,43}\) and changes in domain topology of Nup153 and Nup214 are reflected in this and other immuno-EM studies.\(^{26,27}\) Here, we have shown that the local dynamics of FG-repeat domains can change in response to salts and energy-depletion. All these aspects of NPC dynamics and plasticity, although still poorly understood at the molecular level, are important to consider in the context of constitutive nuclear transport and its modulation in response to specific signaling and cell growth conditions essential for cell survival.

**Materials and Methods**

All experimental procedures were performed at room temperature unless stated otherwise.

**Immuno-EM of isolated nuclei from Xenopus oocytes and labeling at the equilibrium state**

Mature (stage 6) oocytes were surgically removed from female Xenopus laevis, and their nuclei were isolated as described.\(^{44}\) Colloidal gold particles, ~8 nm in diameter, were prepared by reduction of tetrachlorauric acid with sodium citrate in the presence of tannic acid, and antibodies were conjugated to colloidal gold particles as described.\(^{45}\) Isolated nuclei were labeled with anti-Nup153 antibodies as described.\(^{26}\) In the case of Nup214, nuclei were incubated in a solution of anti-Nup214 antibodies diluted 1:1000 in PBS for 2 h and washed twice in PBS. After washing, nuclei were incubated for 2 h in an anti-rabbit IgG antibody
conjugated to 10 nm colloidal gold (BBI International, Cardiff, UK). Labeled nuclei were fixed and processed for EM as described.\textsuperscript{26,27}

**Immunolocalization of FG-repeat domains in the presence of exogenous calcium**

Freshly isolated nuclei from *Xenopus* oocytes were collected in low-salt buffer (LSB; 10 mM Hepes (pH 7.5), 1 mM KCl, 0.5 mM MgCl\(_2\)) and incubated in, for 30 min LSB containing (1) 100 μM Ca\(^{2+}\), (2) 2 mM Ca\(^{2+}\), and (3) 2 mM Mg\(^{2+}\). Next, the nuclei were fixed for 5 min in LSB containing 4% (v/v) formaldehyde. The nuclei were washed twice in LSB for 5 min each time, and incubated in anti-Nup153-C2 antibody conjugated directly to 8 nm gold\textsuperscript{26} for 2 h to determine the localization of the FG-repeat domains of Nup153. For localization of the FG-repeat domain of Nup214, nuclei were incubated in anti-Nup214-D antibody and secondary anti-rabbit-IgG antibody conjugated to 10 nm colloidal gold as described above. After labeling, the nuclei were prepared for EM as described above.

**Immunolocalization of FG-repeat domains after depletion of stored Ca\(^{2+}\)**

Freshly isolated nuclei from *Xenopus* oocytes were collected in LSB and incubated in LSB containing 10 mM EGTA for 10 min, and 1 μM thapsigargin for 30 min. Next, the nuclei were fixed for 5 min in LSB containing 4% formaldehyde. The nuclei were washed twice in LSB for 5 min each time, and incubated in anti-Nup153-C2 antibody directly conjugated to 8 nm gold\textsuperscript{26} for 2 h to determine the localization of the FG-repeat domains of Nup153. For localization of the FG-repeat domain of Nup214, nuclei were incubated in anti-Nup214-D antibody and secondary anti-rabbit-IgG antibody conjugated to 10 nm colloidal gold as described above. After labeling, the nuclei were prepared for EM as described above.

**Immunolocalization of FG-repeat domains under different concentrations of ATP**

Freshly isolated nuclei from *Xenopus* oocytes were collected in LSB and incubated in LSB containing 10 mM ATP for various lengths of time, as indicated. To deplete ATP, isolated nuclei were incubated in LSB containing apyrase (2 units/ml) for 30 min. Next, the nuclei were fixed for 5 min in LSB containing 4% formaldehyde. The nuclei were washed twice in LSB for 5 min each time, and incubated in anti-Nup153-C2 antibody directly conjugated to 8 nm gold\textsuperscript{26} for 2 h to determine the localization of the FG-repeat domains of Nup153. For localization of the FG-repeat domain of Nup214, nuclei were incubated in anti-Nup214-D antibody and secondary anti-rabbit-IgG antibody conjugated to 10 nm colloidal gold as described above. After labeling, the nuclei were prepared for EM as described above.

**Cryo-EM and 2-D image processing**

*Xenopus* oocyte nuclei were isolated, opened manually and spread on an EM grid as described.\textsuperscript{26} After washing the grid with LSB, a 5 μl droplet of LSB containing 100 μM Ca\(^{2+}\) or a 5 μl droplet of fresh LSB was applied and allowed to equilibrate for 15 min. Samples were prepared for rapid freezing and zero-loss filtered EM and image processing essentially as described.\textsuperscript{8} In brief, the images were recorded digitally with a slow-scan CCD camera (Proscan, Scheuring, Germany; 2 MHz read-out, 14 bit information depth, 1024×1024 pixels). The microscope and the camera were controlled by a VIPS-1000 (Tietz Video and Image Processing Systems, Gauting, Germany). The sample thickness was determined on-line from a zero-loss filtered/unfiltered image pair by a macro routine applying the log/ratio method,\textsuperscript{46} combined with the experimentally determined partial inelastic mean free electron path\textsuperscript{37}. All images were recorded at 120 kV acceleration voltage. The magnification was 12,500× (15,600× on the camera), and the defocus was 15 μm. The electron dose ranged between 300 and 500 e\(^{-}/\)nm\(^{2}\), and 2-D image processing was performed as described.\textsuperscript{8} The multivariate statistical analysis was performed with the Coran program package.\textsuperscript{46} From each image data set, ~900 particles in 128×128 pixel subframes were extracted interactively and contrast-normalized.

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2006.08.021

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