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Structure and mechanics of the human nuclear pore complex basket using correlative AFM-fluorescence superresolution microscopy†

Anthony Vial, ^a Luca Costa, ^a Patrice Dosset,^a Pietro Rosso,^a Gaëlle Boutières,^a Orestis Faklaris, ^b Heiko Haschke,^c Pierre-Emmanuel Milhiet ^{*a} and Christine M. Doucet ^{*a}

Nuclear pore complexes (NPCs) are the only gateways between the nucleus and cytoplasm in eukaryotic cells. They restrict free diffusion to molecules below 5 nm while facilitating the active transport of selected cargoes, sometimes as large as the pore itself. This versatility implies an important pore plasticity. Recently, cryo-EM and AI-based protein modeling of human NPC revealed with acute precision how most constituents are arranged. But the basket, a fish trap-like structure capping the nucleoplasmic side of the pore, remains poorly resolved. Here by atomic force microscopy (AFM) coupled to single molecule localization microscopy (SMLM) we revealed that the basket is very soft and explores a large conformational landscape: apart from its canonical basket shape, it dives into the central pore channel or opens, with filaments reaching to the pore sides. Our observations highlight how this structure can adapt and let morphologically diverse cargoes shuttle through NPCs.

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Introduction

In eukaryotic cells the nuclear envelope (NE) isolates the genome from the rest of the cell. This physical barrier is composed of two biological membranes: the outer nuclear membrane (ONM), that is continuous with the endoplasmic reticulum, and the inner nuclear membrane (INM), which harbors a unique integral protein composition.¹ Nuclear pore complexes (NPCs) are the only gateways between the cytoplasm and nucleoplasm. Their primary function is to ensure the permeability barrier: they restrict passive diffusion to small molecules below 40 kDa, yet allowing regulated transport of larger protein assemblies, including the export of mRNAs.² Very large MDa molecular assemblies can be transported through NPCs in their native state, such as proteasomes or viral capsids for instance.^{3–6} As a matter of fact, NPCs are the largest protein complexes found in eukaryotic cells, with a diameter around 100 nm in humans.^{7–10} They are composed of over 1000 polypeptides for a total size of 110 MDa in humans.⁷ With their 8-fold rotational symmetry, NPCs are composed of about 30

different proteins called nucleoporins (Nups), present in multiples of 8 copies per pore. The NPC scaffold is made of three stacked rings embedded in the NE, and a luminal ring located in the perinuclear space. The central channel is filled with nucleoporins rich in FG-repeats whose physico-chemical properties rule the permeability barrier.^{11,12} This whole structure is capped by cytoplasmic filaments on one side, and a nuclear basket on the other (Fig. 1A).

Years of intense structural investigations have gradually revealed the molecular architecture of the human NPC scaffold. Cryo-electron microscopy (cryo-EM) performed on purified NPCs or *in situ* has depicted the arrangement of the luminal, inner, cytoplasmic and nucleoplasmic rings with respect to the NE membrane, at resolutions down to 2 nm.^{13–17} Integrative structural studies have positioned X-ray crystallographic and model structures of individual Nup fragments within the electron-dense volumes. We now visualize with sub-nanometer resolution how Nups are arranged within the scaffold.^{8,13,15–19} In striking contrast, the organization of cytoplasmic filaments and nuclear basket in human cells is not well solved in cryo-EM maps. One interpretation is that these domains are highly flexible, leading to a continuum of conformations that cannot be resolved by cryo-EM. While a near atomic composite model of NPC cytoplasmic face was recently published,¹³ the nuclear basket is notoriously missing. From scanning electron micrographs taken in the 90 s of *Xenopus laevis* nuclear envelopes,²⁰ we know that the basket is made of

^aCBS (Centre de Biologie Structurale), Univ Montpellier, CNRS, INSERM, Montpellier, France. E-mail: pem@cbs.cnrs.fr, christine.doucet@cbs.cnrs.fr

^bMRI, Biocampus, University of Montpellier, CNRS, INSERM, Montpellier, France

^cBrüker Nano GmbH, Berlin, Germany

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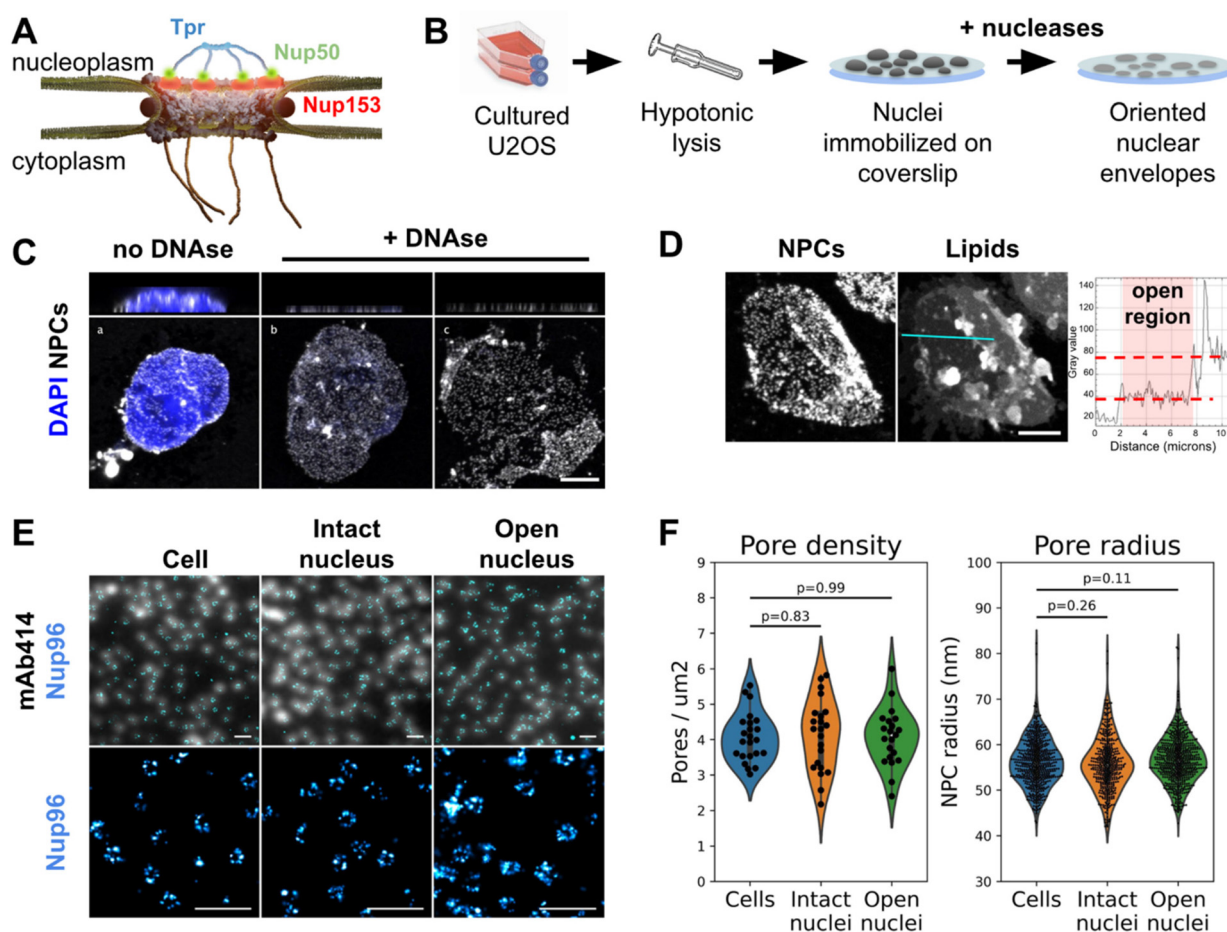


Fig. 1 The nuclear membrane is preserved during NE preparation. (A) Schematics of the human nuclear pore complex, with emphasis on basket organization. (B) Schematic procedure for nuclear envelope preparation from cultured U2OS. (C) 3D-confocal imaging of nuclei isolated from U2OS and treated or not with nucleases. The chromatin was labeled with Hoechst (blue) and NPCs with WGA (white). Top panels show xz sections; bottom panels show the lower nuclear membrane. Scale bar is 5 μ m. (D) Confocal imaging of an open nucleus. NPCs are labeled with WGA-AF594, lipids with DiOC6. Scale bar is 5 μ m. The right panel shows the intensity profile of the lipid dye along the cyan section. (E) Nuclei and nuclear envelopes were extracted from U2OS/Nup96-SNAP. Intact cells, intact nuclei or open nuclei were labelled with mAb414-AF594 and AF647-SNAP ligand. Diffraction-limited (mAb414) and dSTORM images (Nup96-SNAP) were acquired in TIRF illumination; scale bars are 2 μ m (top) and 500 nm (bottom). (F) Pore density was measured from confocal images (shown in Fig. S2C,† $n > 20$ nuclei); NPC radii were measured from dSTORM images as exemplified in E ($n \geq 3$ nuclei).

eight filaments attached at their base to the NPC scaffold and joining together at their distal ends. The resulting structure appeared as a fish trap-like net, hence its name – the basket. In higher eukaryotes, this structure is mainly composed of three Nups: Nup153 and Nup50, located at the base of the basket, and Tpr which contains four predicted coiled-coil domains and is the major constituent of the filaments.²¹ During their nucleo-cytoplasmic shuttling, cargoes have to pass through the basket, which caps the nucleoplasmic opening of the channel. It is hard to envision how very large cargoes, such as intact viral capsids,⁵ can make their way in this intricate structure. Indeed, such large cargoes almost fill the entire space within the central pore channel, and their release in the nucleoplasm must involve some opening or disassembly of the basket. As a matter of fact, while most components of the scaffold are very stably associated, several Nups

nevertheless can exchange from the NPC.²² It is in particular the case for Nups that connect core NPC components together²³ and their high exchange rate was proposed to favor structural adaptation of the NPC during cargo translocation for instance. Likewise, components of the basket are highly dynamic: they exchange rapidly with the NPC, indicating a continuous turnover between NPC-bound and free Nups.^{22–25} Moreover, serial analysis of thousands of NPCs imaged by single molecule localization microscopy (SMLM) show that the main basket constituent, Tpr, exhibits a blurry spatial distribution while other components show a better-defined localization.²⁶ However, most studies cited above rely on ensemble or averaged structural data. It is thus not clear how structural dynamics and/or variability of the basket translate at the single pore level. Therefore, to explore different basket conformations, we used atomic force microscopy (AFM), that does not rely on averaging.

Thanks to its very high signal to noise ratio, AFM provides sharp topography images of single particles and operates in liquid, making it an asset to study heterogeneous samples in physiological conditions.²⁷ In AFM, samples are probed by a nanometric tip located at the end of a cantilever. Tip-sample interactions induce deflection of the cantilever, which can be detected and analyzed to reconstruct the topography of the sample surface and can also provide mechanical information. Furthermore, combined with super resolution fluorescence microscopy, molecular mapping can be correlated with AFM topography of biological membranes with lateral resolutions up to 10–30 nm (see a review in ref. 28 and a study carried out in our group²⁹). AFM thus appears as a method of choice to observe and describe at high resolution the conformational states of NPCs. However, this technique requires direct accessibility to the probed surface. In the context of NPC study, it means to isolate nuclei and purify NEs. This technique has been developed on *X. laevis* stage-VI oocytes, since their nuclei are large enough (~300 μm in diameter) to be manually teased open.³⁰ High resolution images of the cytoplasmic and nucleoplasmic sides of *Xenopus* NPCs have revealed a certain degree of architectural plasticity – their morphology changes in response to different physiological stimuli or addition of transport receptors.^{25,31–38} However, these works did not provide an extensive description of the conformational landscape of NPC baskets. In other words, it is not clear whether baskets can adopt a finite number of conformations or freely explore a continuum of conformations. Considering the crucial role of NPCs in cellular homeostasis and the fact that they are associated with several diseases,^{39–42} there is a real interest in elucidating the structure of human nuclear pores and understanding their plasticity. Indeed, even though NPCs are highly conserved among eukaryotes, there are compositional and/or structural differences between species.^{14,43} Moreover, oocytes are in a particular metabolic state, and this translates into structural and functional specificities even in NPC organization: NPCs are ~10 times denser than in somatic cells, and their structure and size evolves during gametogenesis.⁴⁴ A few studies have been performed on biochemically extracted human NEs^{45,46,81,82} but none of them focused on NPC baskets.

We established a biochemical procedure to open nuclei isolated from cultured human U2OS cells and imaged by AFM the nucleoplasmic face of hundreds of NPCs in their physiological environment. Thorough statistical analysis of AFM images showed that the basket largely explores the pore central channel rather than systematically protruding into the nucleoplasm. We also located Tpr within the basket by correlating AFM with super-resolution microscopy and found pores with open baskets, *i.e.* with Tpr-containing filaments reaching towards the membrane around the pore. Comparing the topography of normal NPCs and NPCs devoid of basket demonstrated that the basket contributes to an important volume in the central channel of NPCs, and to a lesser extent at the surface of the scaffold rim. Finally, we found that the basket filaments are very soft, an undeniable asset for structural plas-

ticity. Our findings explain why cryo-EM cannot currently decipher the basket structure but most importantly, they enlighten how it can adapt to cargoes of different morphologies and provide a flexible frame to support nucleo-cytoplasmic transport.

Results

Preparation of human nuclear envelopes (NEs) with preserved nuclear membrane organization

It appeared recently that biochemically purified NPCs are narrower than their counterparts imaged *in situ*.^{8,14} This is arguably due to a loss of tension in the NE. To avoid this artifact, we adapted a classical biochemical procedure to purify NEs,⁴⁷ applying it to nuclei immobilized on glass in order to preserve their spatial organization, orientation and tension. Briefly, a hypotonic cell lysis is carried out using a Dounce homogenizer. The purified nuclei are immobilized on glass coverslips, then treated with a mixture of RNase and DNase in a hypotonic buffer (Fig. 1B, see Methods for more details). A fraction of nuclei spontaneously opens during this step (Fig. 1C). As nuclei are immobilized on glass prior to treatment, when they open, the top NE sheet is removed, leaving the bottom layer intact with its INM facing up. Samples were then fixed and fluorescently labeled for correlative AFM/fluorescence studies. Since nuclei are open, labeling can be performed without detergent, keeping the nuclear membranes intact. We demonstrated that the successive biochemical treatments indeed preserve the nuclear envelope and nuclear pores. While nuclease treatment completely digests chromatin in most nuclei (Fig. S1A†), it results in different configurations: some nuclei remain apparently intact (Fig. 1C-b) while a majority have a fragment of NE ripped off (Fig. 1C-c). The areas where one or two layers of NE are present are easily delineated thanks to the two-fold difference in NPC density (Fig. 1C and S1B†) or lipid staining (Fig. 1D). Some nuclei expose a well circumscribed central hole; in these cases the membrane organization is well preserved, based on the homogenous lamin-B1 staining (Fig. S1C-e and f†) and further confirmation by AFM imaging (Fig. 2A & B for instance). These nuclei, easily recognizable, were selected for further studies. On the contrary, some nuclei have large sections of NE ripped off; in those the organization of INM was disrupted, as shown by lamin staining (Fig. S1C-c and d†), and they were discarded.

Regarding NPC organization, pore densities measured from cultured cells and purified nuclei – treated or not with nucleases – are similar (Fig. 1F & S1B†). Moreover, direct stochastic optical reconstruction microscopy (*d*STORM) imaging on NPCs allowed us to measure their diameters (Fig. 1E and F). For this, we used a modified U2OS cell-line whose endogenous nucleoporin Nup96 is fused to a SNAP-tag.⁴⁸ Nup96 is a main constituent of NPCs scaffold and its distribution is thus representative of the nuclear pore structure. We labeled intact cells and nuclei – intact or open – with an AlexaFluor 647 SNAP-ligand and performed *d*STORM imaging. The diameter of Nup96-SNAP rings measured in the three situations shows



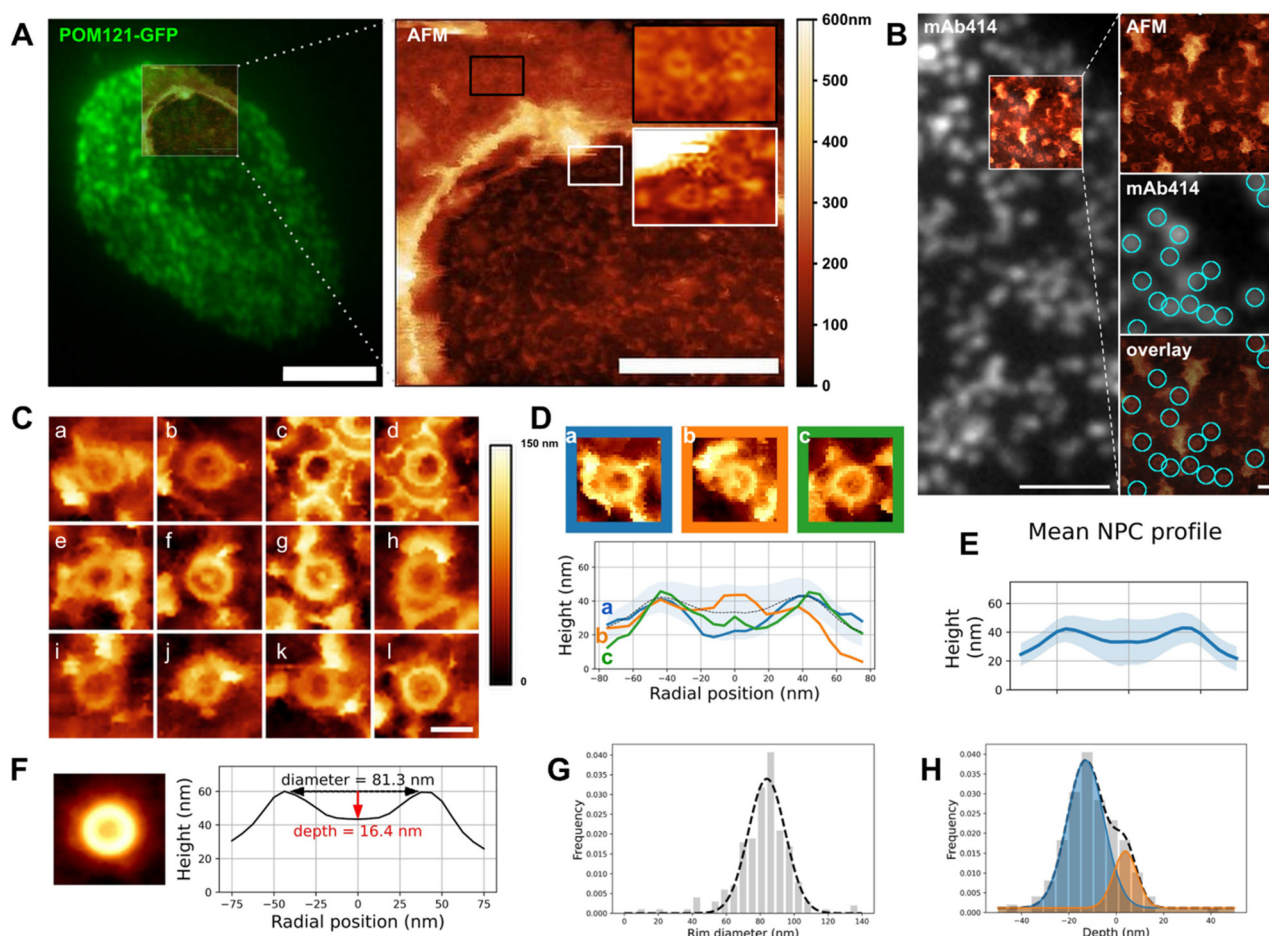


Fig. 2 Structure of the NPC basket. (A) Correlative fluorescence/AFM image of an open nucleus isolated from U2OS overexpressing POM121-GFP. Left: TIRF image of the entire nucleus overlaid with the region scanned by AFM. Right: Height image acquired by AFM, encompassing the opening border. Scale bars are 5 μm (left), 2 μm (right). (B) Correlative TIRF/AFM image of the inner nuclear envelope of an open nucleus. NPCs are labelled with mAb414 and visualized as fluorescent dots that coincide with ring-like structures in the AFM image. Scale bars are 2 μm and 200 nm. (C) Representative samples of human nuclear pores (nucleoplasmic face) imaged by AFM. Scale bar is 100 nm. (D) Three configurations of NPC nucleoplasmic region and their respective height profile. "Empty" (blue), protruding (orange) and low basket (green). (E) Mean of rotationally averaged NPC height profiles ($n = 210$). The shaded area represents the standard deviation. (F) (Left) Average image of 210 NPC crops. AFM color scale as above. (Right) Height profile of the resulting image. (G and H) Diameter and depths distributions of 210 NPCs plotted as frequencies and fitted with one (respectively two) Gaussians. The dashed line represents the fit function. These graphs are also shown in Fig. S2D & E[†] to illustrate the analysis workflow.

no significant differences (Fig. 1F). Conservation of pore density and diameter suggests that adhesion of the NE prior to its rupture prevents NPC remodeling observed in biochemical NE fractions.⁸ In conclusion, we developed a method to prepare flat human NEs, free of chromatin, with areas of INM accessible to the AFM tip.

Topography of the NPC basket

The notorious absence of the pore basket in averaged structures requires assessing the nucleoplasmic topography of individual pores. Documenting the conformational landscape of the basket can reveal how rapid exchange of Tpr and Nup153 translates in the basket structure. Moreover, this will elucidate to which extent the pore basket can deviate from the canonical fishtrap-like structure into conformations compatible with

transport of large cargoes. Fig. 2A shows an open nucleus prepared from U2OS cells expressing the transmembrane nucleoporin POM121 fused to GFP (POM121-GFP). Based on pore density, we assessed that this nucleus had a central, well-delineated hole as previously discussed. AFM imaging confirms the presence of this hole surrounded by a bulge of membrane (Fig. 2A, right panel). Nuclear pores are clearly delineated by the AFM tip on both the cytoplasmic (black inset) and nucleoplasmic faces (white inset). In order to probe the basket structure, we then focused on the INM. NEs were routinely prepared and labeled with a widely used fluorescent antibody against NPCs recognizing several Nups (mAb414 conjugated to Alexa Fluor 594). The fluorescent pore signal correlates with ring structures in the AFM image (Fig. 2B). 250 nm crops around each pore were extracted (examples shown in Fig. 2C) and a



Altogether our results show that, while the ring structure is rather conserved from pore to pore, the central region corresponding to the basket is extremely variable and a majority of basket structures do not protrude into the nucleoplasm but rather reside in the central pore channel. Considering that Tpr is a main component of the basket filaments,²¹ a quantitative evaluation of Tpr organization within pores, especially when an empty ring is observed, was performed.

Tpr is a 267 kDa protein that contains 4 coiled-coil regions in its N-terminal part while its C-terminus is mostly disordered. Coiled-coils are helical domains that can oligomerize and constitute rigid molecular rods.^{49,50} In the case of Tpr, the coiled-coils are interleaved with flexible linkers. Tpr thus contains the structural ingredients to provide filamentous yet articulated segments, which may be an asset for the basket to adapt its shape to cargoes.

These images are reminiscent of the three NPC conformations described previously (Fig. 2D) and further document the fact that the NPC basket can adopt various configurations. Importantly, as *d*STORM imaging was performed before AFM imaging, the asymmetry observed in Tpr organization in some NPCs is not due to deformation by the AFM tip. However, it could be due to deformations that arose during the biochemical preparation. We thus explored the basket structure in intact cells by 2-color STED (stimulated emission depletion) microscopy. This confocal-based super-resolution microscopy technique is highly convenient for multi-colour imaging and achieves a typical lateral resolution in fixed biological samples around 40 nm.^{52,53} We performed co-immuno-fluorescence in fixed cells against Tpr (coupled to a Star-635P fluorophore) and Nup153 (coupled to an Alexa Fluor 594) to visualize their relative organization. As a control, we used Nup153 co-labeled with Star-635P and Alexa Fluor 594; as expected, Nup153 imaged in both channels renders a very similar signal. Conversely, Elys, which is one of the outmost nucleoporins,²⁶ is localized as a rim around Nup153 central dot, with some variability in the labeling efficiency (Fig. 3D). Regarding Tpr, it usually appears as one – sometimes two – dots that can be well aligned with Nup153 signal or slightly shifted (Fig. 3D, middle row). To characterize the relative positioning of these various

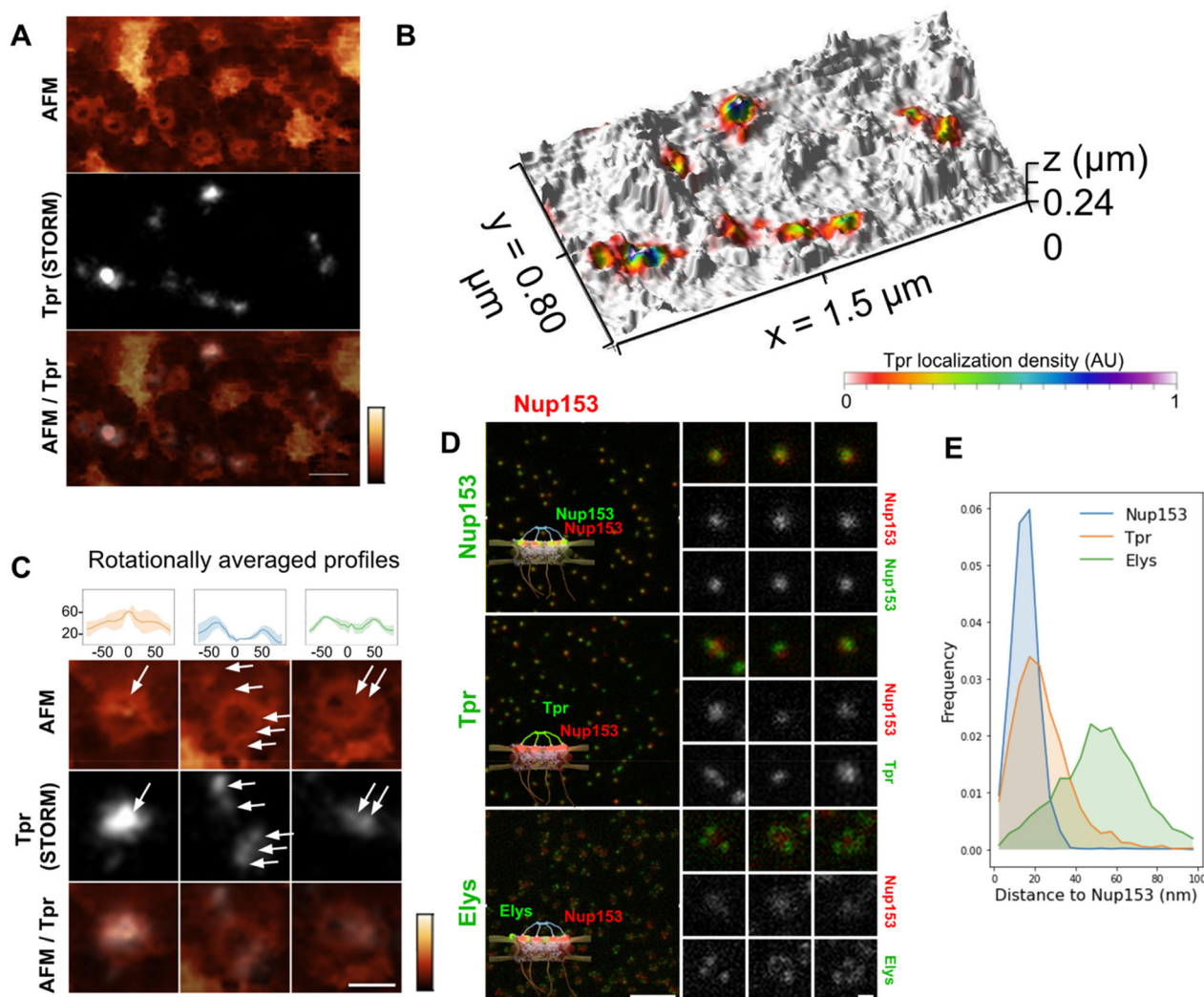


Fig. 3 Tpr organization within the NPC basket. (A) Correlative AFM/dSTORM image of an open nucleus prepared from U2OS cells. The sample was immuno-labelled against Tpr. dSTORM imaging was performed in TIRF illumination. The same area was then imaged by AFM. After reconstruction of the dSTORM localizations map, the two images were correlated (scale bar is 200 nm). (B) 3D representation of the correlated AFM/dSTORM image. (C) Three NPCs of typically different topographies are shown in more details, together with their rotationally averaged height profiles (upper panel). Arrows point at Tpr localizations (scale bar is 100 nm). (D) U2OS cells were fixed and simultaneously immuno-labelled against Nup153, Tpr or Elys, with a secondary antibody coupled to STAR-635P (green) and Nup153 coupled to AlexaFluor 594 (red). Samples were imaged by STED microscopy. Individual NPCs are zoomed on the right (scale bars are 1 μ m and 100 nm). (E) Distance distribution between the AlexaFluor 594 and STAR-635P signals measured from STED images (number of pores >2700).

proteins, we measured distances between Nup153 labeled with Alexa-594 and Nup153, Elys or Tpr labeled with Star-635P (Fig. 3E). The distribution shows that a fraction of Tpr is shifted away from Nup153, towards the nucleoplasmic ring where Elys resides. These results obtained on entire cells confirm the variability and asymmetry of the NPC basket observed in AFM images.

Altogether, our AFM and super-resolution microscopy data demonstrate important basket plasticity in the human NPC: Tpr-containing filaments can either protrude towards the nucleoplasm, collapse within the channel or partially disassemble at the level of the distal ring, with filaments pointing outwards from the pore channel axis.

Contribution of the nuclear basket in the topography of NPCs

The presence of interleaved disordered linkers between its coiled-coil domains presumably allows Tpr to explore a large range of conformations, from a completely extended to a rather globular shape – as predicted by AlphaFold for instance.⁵⁴ It is thus hard to predict how deep the basket could collapse into the pore channel. To understand the contribution of the basket in the overall NPC structure, we imaged pores in open nuclei depleted from Nup153. Indeed, as reported previously,⁵⁵ siRNAs against Nup153 prevent Tpr binding to NPCs. Triple labeling of Nup153, Nup96, and Tpr (Fig. 4A) confirms that Nup153 depletion is associated with

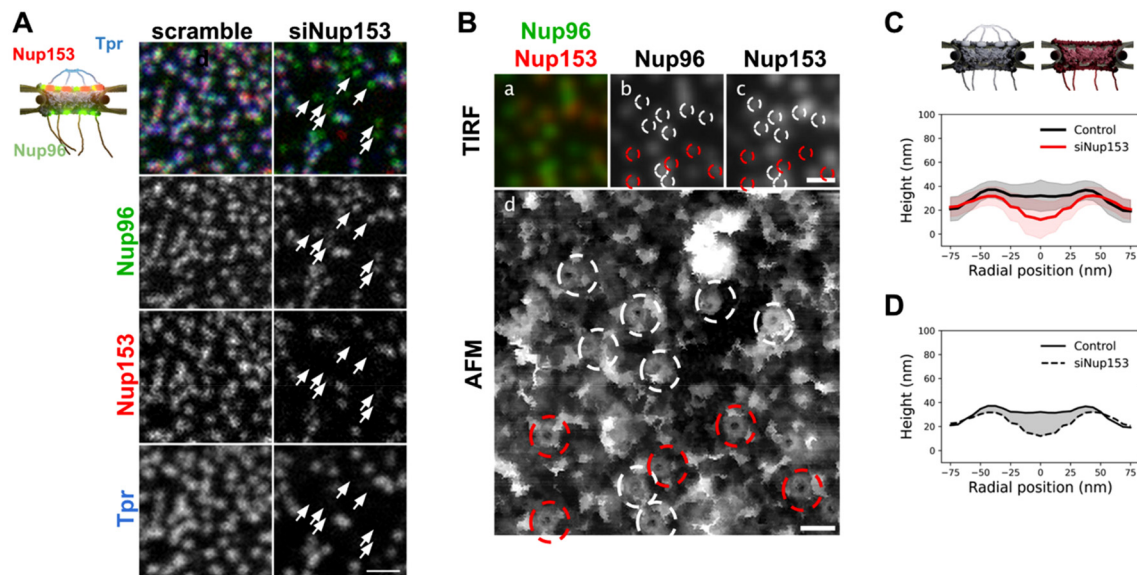


Fig. 4 Basket contribution in the topography of human NPCs. (A) Stable U2OS cells expressing Nup96-GFP were transfected with siRNAs, scrambled or targeted against Nup153. Cells were then fixed, co-labeled with anti-Nup153 (red) and anti-Tpr (blue) and imaged by confocal microscopy. Arrows indicate pores depleted of Nup153. They are consistently co-depleted of Tpr (scale bar is 1 μ m). (B) Correlative AFM-fluorescence image of a NE prepared from U2OS/Nup96-GFP cells depleted of Nup153 by siRNA and labeled with anti-Nup153. Panel d shows the ROI scanned by AFM. Top panels show the corresponding Nup96 (b) and Nup153 (c) channels imaged by TIRF. In panels b–d, NPCs are circled in white when Nup153 is detected and in red when it is absent or weak. Scale bars are respectively 500 nm (a–c) and 200 nm (d). (C) Rotationally averaged height profiles were averaged from over 80 pores imaged from control (black) and siNup153-treated cells (red). Shaded areas are standard deviations. (D) The average contribution of the basket in the NPC structure can be envisioned as the volume located between the average surface of control NPCs and of basket-depleted NPCs. A cross-section of this volume is represented as the grey shaded area.

the absence of Tpr, while Nup96 remains visible (Fig. 4A, arrows point at pores without Nup153 or Tpr). It is important to note that siRNAs against Nups only achieve partial depletion.⁵⁶ In the case of Nup153 depletion, it results in a mild decrease in pore density (Fig. S4A†) and importantly, only a fraction of the remaining pores are completely devoid of Nup153 (Fig. S4B†), which complicates the interpretation of topological differences observed between depleted and naive nuclei.

In nuclei with co-labeled Nup96 and Nup153, we can distinguish in correlative AFM/fluorescence images pores that contain Nup153 (circled in white in Fig. 4B) or not (circled in red). In the corresponding AFM image, a central topography is more frequent in Nup153-associated NPCs. In contrast, a deep hole is more frequently observed in pores devoid of Nup153. Importantly, even when Nup153 is detected, its levels seem to vary from pore to pore but it is in all cases lower than in mock-depleted cells. To quantify how Nup153 depletion affects NPC topography, we generated average height profiles for pores imaged from Nup153-depleted NEs and compared to naive pores (Fig. 4C). Average height measured at the center is dramatically decreased when Nup153 is depleted. The height standard deviations (shaded areas in Fig. 4C) partly overlap, reflecting that some pores from depleted cells have similar topographies as compared to control pores. This overlap is likely due to remaining levels of Nup153. One can nevertheless note that very few Nup153-depleted NPCs were seen with protruding baskets (Fig. S4C†). This conformation may require high levels

of Nup153 and Tpr, which is very unlikely to occur in depleted cells. Finally, the very low topographies observed in depleted nuclei probably reflect a total absence of basket. In this case, the AFM tip most likely probes the central FG-repeats. Variability of the central topography may reflect plasticity of the central channel FG-rich nucleoporins. Not to forget also that the central channel is partially occupied by shuttling cargoes, which may contribute to the overall topography and add up to the inherent variability.¹⁹ Anyway, this height difference between control and basket-depleted pores confirms that in most cases, even when the basket shape is not clearly depicted by AFM, it accounts for some molecular density occupying part of the central channel. This average contribution is visualized in Fig. 4D, as the shaded area. The height of the scaffold ring is also lower when Nup153 is depleted. This height difference reflects that the basket sits on top of the scaffold. Nup153 is probably present at this location since it interacts with the nucleoplasmic ring.^{24,57} Nup153 is mostly intrinsically disordered⁵⁸ and its structure is likely more fuzzy than the highly ordered components of the nucleoplasmic ring.⁸ This could explain the important volume occupancy at the top of the scaffold ring.

Mechanical properties of the basket

Altogether, our data show that the basket can adopt a large range of conformations. We wondered whether this important structural variability is related to mechanical flexibility. AFM is



Since the basket, which is already very soft, appears suspended on top of an even softer medium, we wondered if it could be deflected by the AFM tip during imaging, which would affect the topographical images. Therefore we reconstructed the sample height with different forces applied (Fig. S5B†). Although a small indentation is visible with increasing forces, the overall conformation of the basket remains unchanged.

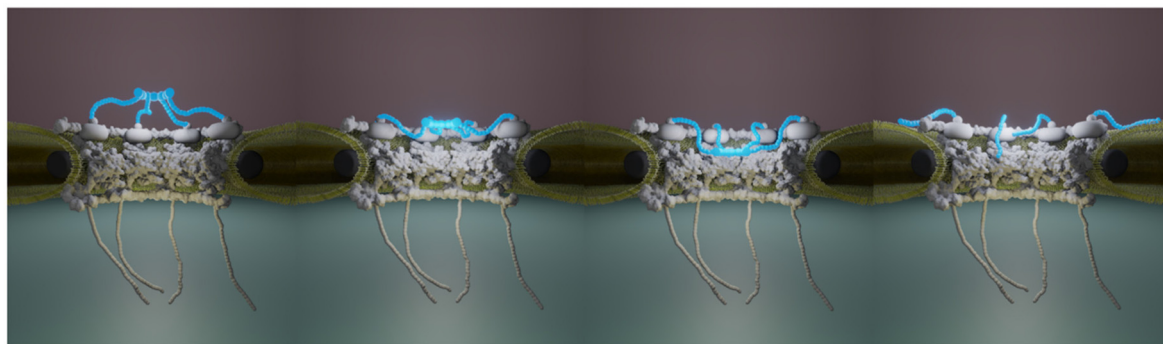


Fig. 6 NPC basket variability. This cartoon illustrates a subset of conformations that the basket (in blue) can adopt. The mechanical softness of the basket allows the Tpr-containing filaments to explore a continuum of conformations from an extended configuration, protruding into the nucleoplasm, to a collapsed conformation where the filaments dive into the central pore channel. In addition, the filaments distal ends can dissociate, leading to an open conformation.

indicates that the basket accounts for roughly 13% of the total NPC mass. Yet we show that it can explore a large volume (Fig. 4D). Consequently, the electron density from the basket might well fall below the detection limit in cryo-EM.

These considerations underline the unique possibilities offered by AFM, which does not require averaging and provides mechanical information. As a matter of fact, NPCs have been widely studied by AFM in NEs extracted from *X. laevis* oocytes. These studies documented pore diameters around 81–85 nm for the cytoplasmic side^{25,32–34,65} and 80–88 nm at the nucleoplasmic side when measured at the ridge.^{32,33,65,66} The mean diameter that we measured here from human INM – 81.5 ± 16.3 nm – thus falls in the same range. Importantly, these studies also showed great variability of the basket conformation (protruding baskets and hollow pores). Moreover, some of this work showed that nuclear transport receptors⁶⁵ or calcium^{36,37,67} induced conformational changes of the basket. Here, from unperturbed conditions, we observed various basket conformations, which may indeed reflect pores engaged in transport of different cargoes for instance. Fig. 2D illustrates three obviously different conformations, but it appeared rapidly that baskets can adopt many more conformations, suggesting it can explore a continuous conformational landscape. Quantification of the topography of hundreds of nuclear pore baskets performed in our study strongly suggests that the position of the highest region of the basket can protrude above the scaffold or collapse into the central channel with positions covering a continuum of distances of 60 nm (Fig. 2H), the basket exploring a continuous space within the central channel up to the nucleoplasm. Importantly, using correlative AFM/dSTORM microscopy appears here as an important asset to specifically assign Tpr fluorescence signal to structures delineated by the AFM tip. This let us unambiguously assign the basket structure, and was especially helpful in identifying unexpected conformations such as collapsed or open baskets (Fig. 3C).

Although it is tricky to compare data from different publications, we noticed that samples subjected to stronger fixation conditions (such as 1–2.5% glutaraldehyde) exhibit more pro-

truding baskets,^{20,21} while mild (4% PFA for 5 minutes in this study) or absence of fixation^{32,37,65} leads to more hollow baskets. This suggests that chemical fixation, which is known to rigidify samples,^{68,69} may preclude pore plasticity. As a matter of fact, we and others^{19,32} showed that the basket is extremely soft and observed high standard deviation for stiffness values underlining the large mechanical variability in the central region of pores. The origin of this variability is not clear but may reside in the transport status of pores: indeed, the density and size of cargoes located in the central channel may influence the stiffness measured in this area. Of note, stiffness values given in previous work are higher than what we measured in human NPCs, which can be explained by the use of higher frequency (kHz) in PeakForce QNM by Stanley *et al.* This can overestimate the stiffness as compared to our quasi-static frequency range (typically 10–100 Hz for QI). As a matter of fact, supplementary control experiments performed by Stanley *et al.* using force volume, which probes the sample at lower speed, exhibit much lower stiffness values, closer to our results.³²

Altogether these observations suggest that mechanical flexibility is the key parameter that allows the basket to explore a continuum of conformations. Being less restricted than other parts of the nuclear pore, the peripheral domains (cytoplasmic filaments and basket) require a lower entropic cost to change conformation. In this regard, they may be an asset for NPCs to adjust their shape. In addition to this deformation scenario, individual filaments may rapidly exchange from NPCs and reassemble in different configurations. This is supported by data showing the high exchange rate of Tpr and Nup153.^{22–24} In particular, Nup153 dynamics may destabilize Tpr association to the scaffold and loosen filament attachment. This is in agreement with many of our individual NPC images where the basket appears asymmetrical both in terms of topography and Tpr localization (Fig. 3). It was also shown recently in budding yeast that peripheral NPC components are loosely linked to the main scaffold and their interactions with the pore are labile.²³ In this study the authors suggest that the dynamic nucleoporins could be envisioned as “a cloud of accessory factors surrounding and constantly exchanging with



All antibodies and fluorescent labels used in this study are commercially available. The purchase references and dilutions used are listed in Table 1.

Cells were cultured in DMEM/Glutamax (Gibco) supplemented with 10% fetal calf serum (Gibco) in a humid atmosphere at 37 °C, 5% CO₂.

For siRNA transfection, 1.5 millions of cells were seeded in 10 cm diameter plates. The day after they were transfected with 1.25 nmol of siRNA and 10 μ L of Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Medium was changed after 4–6 hours. Transfection was repeated 48 h later in identical conditions. Cells were lysed or imaged the day after.

All buffer compositions are indicated in Table 2. Nuclei were prepared from cultured cells as previously described.⁴⁷ Briefly, around 40 millions cells (four 75 cm² confluent flasks) were trypsinized. Cells were rinsed in PBS then hypotonic buffer (HB) by 5 minutes centrifugation at 300g. Cells were then resuspended in 1 mL HB, incubated on ice for 45 minutes and disrupted by mechanical shearing using a Dounce with a tight pestle (approximately 10 strokes are sufficient). Cell lysis was controlled by visual inspection using an inverted microscope (Primovert, ZEISS). The nuclei suspension was then loaded on top of 5 mL of S500 and spun for 15 minutes at 300g. The supernatant containing cellular debris was discarded, the pellet was resuspended in 300 µl of S500, transferred to a clean tube containing 5 mL of S500 and spun for 3 min at 300g. The pellet, containing mostly intact cells, was discarded. The supernatant was transferred to a clean tube and spun for 15 minutes at 300g. Nuclei were resuspended in S500 at a final density of 10⁶ nuclei per mL.

Cell strains, antibodies and siRNA

U2OS cells were obtained from ATCC. CRISPR-engineered Nup96-SNAP U2OS cell lines⁴⁸ were obtained from CLS

Reagent	Company	Reference	Dilution
mAb414	Covance		1 : 2000
mAb414 conjugated to AlexaFluor 594	BioLegend	682202	1 : 1000
Anti-lamin B1	Thermo Scientific	PA5-19468	1 : 500
Anti-Nup153 [SA1]	Abcam	ab96462	1 : 200 for STED
anti-Tpr	Abcam	ab84516	1 : 200 for STED and <i>d</i> STORM
Anti-ELYS	Abcam	ab53540	1 : 200 for STED
WGA conjugated to Alexa Fluor 594	Invitrogen	W11262	1 $\mu\text{g ml}^{-1}$ from a stock solution prepared at 5 mg ml^{-1} in DMSO
DiOC6	Invitrogen	D273	1–5 μM from a 5 mM stock solution prepared in DMSO
Alexa Fluor-647 SNAP ligand	New England BioLabs	S9136S	1 μM for <i>d</i> STORM
Alexa Fluor-647 anti-mouse	Molecular probes		1 : 2000
Anti-rabbit or anti-mouse conjugated to Star580, StarRED or Star635P dyes	Abberior instruments		1 : 200 for STED

Buffer	Composition	Ref.
Hypotonic buffer	50 mM Tris pH 7.5, 1 mM DTT, Complete (Roche)	Ori <i>et al.</i> , 2013 ⁴⁷
S500	10 mM HEPES pH 7.5, 2.5 mM MgCl ₂ , 50 mM KCl, 500 mM sucrose, 1 mM DTT, Complete	Doucet <i>et al.</i> , 2010 ⁵⁶
NE-A	0.1 mM MgCl ₂ , 1 mM DTT + 30 U DNaseI (Sigma, D5319) + 250 U RNase A	Ori <i>et al.</i> , 2013 ⁴⁷
NE-B	20 mM Tris pH 8.5, 10% sucrose, 0.1 mM MgCl ₂ , 1 mM DTT	Ori <i>et al.</i> , 2013 ⁴⁷
Nuclei buffer	10 mM HEPES pH 7.5, 2.5 mM MgCl ₂ , 83 mM KCl, 17 mM NaCl	
Immunofluorescence (IF) buffer	PBS wit 10 mg ml ⁻¹ BSA, 0.1% Triton X-100, 0.02% SDS	Doucet <i>et al.</i> , 2010 ⁵⁶
Permeabilization buffer (PB)	PBS with 0.4% Triton X-100	Thevathasan <i>et al.</i> , 2019 ⁴⁸
Quenching solution (QS)	100 mM NH ₄ Cl in PBS	Thevathasan <i>et al.</i> , 2019 ⁴⁸
dSTORM buffer	PBS supplemented with 10% glucose, 0.5 mg mL ⁻¹ glucose oxidase (Sigma), 0.04 mg mL ⁻¹ catalase (Sigma, C3556), 35 mM mercaptoethylamine (MEA).	Thevathasan <i>et al.</i> , 2019 ⁴⁸

Staining with SNAP-ligand was performed for 2 h. Samples were then washed 3 times with PBS for 5 min, and post-fixed in PBS + 4% PFA for 5 minutes. All incubations were performed at room temperature. For imaging, the coverslips were set in an AttoFluor cell chamber and covered with 1.2 mL of *d*STORM buffer (Table 2), covered by a glass coverslip to limit oxidation. The buffer was renewed after 2 hours.

25 mm high-precision type 1.5 glass coverslips of 0.17 mm thickness (Marienfeld) were sequentially washed in acetone, ethanol and water, then sonicated in 1 M KOH for 20 minutes. Coverslips were then extensively washed in milliQ water and

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