Nuclear pore complexes (NPCs) are huge macromolecular assemblies that span the inner and outer nuclear membranes and facilitate nucleocytoplasmic exchange of macromolecules. Due to their enormous size of about 120 nm in diameter, elucidating their structure is an enormous challenge. In the last decade, X-ray crystallography has provided major insights into the fold organization of various nucleoporins and the structure of many protein interfaces.1 It has become clear that scaffold Nups are organized in a modular way and share α-helical repeat and β propeller domain folds.2 Electron microscopy (EM) has revealed the overall shape of some of the subcomplexes, in particular the yeast Nup84 subcomplex.3,4 Taken together with structural modeling approaches, X-ray structures could be localized within the EM map of the yNup84 subcomplex.4,6 Such information, taken together with biochemical data, was integrated into models of the overall architectural organization of the NPC (reviewed, e.g., in ref. 1). Although electron tomography had previously provided density maps of fully assembled NPCs, structures of subcomplexes could not unambiguously be assigned into the structural framework. We have recently generated a tomographic map of the human NPC that is sufficiently resolved to detect structural signatures of larger subcomplexes.7

A three-dimensional map was generated from cryo electron tomograms of purified HeLa cell nuclear envelopes that were analyzed by subtomogram averaging (Fig. 1A). It shows that the nuclear and cytoplasmic rings of the nuclear pore are elevated from the outer and inner nuclear membranes by about 10 nm. Both rings are connected to the membrane by two rod-shaped elements per asymmetric unit (Fig. 1B). Within each ring and asymmetric unit, these elements are positioned very close to each other. All four of these emanate into a Y-shaped element that we refer to as two pairs of the outer and inner vertices, respectively due to slightly different distances from the center. These show significant similarity to the structure of the isolated hNup107 subcomplex (Fig. 1C).

Another rod shaped element per asymmetric unit connects each, the
cytoplasmic and nuclear ring, to the spoke ring. This connector element touches the membrane right at its merging point into the spoke ring (Fig. 1A). One might speculate that Nup53 is positioned there, while the adjacent shapes could be attributed to Nups 155 and 205 (Fig. 1A). Such an assignment, although intriguing, is however speculative at this point, since the crescent shapes typical for Nups 205 and 188 are observed in multiple orientations and positions throughout the entire structure and the entire mid-plane of the spoke ring, which contributes a considerable amount of density, would remain unexplained.

The improved resolution of the tomographic structure was primarily enabled by the development of automatized data acquisition protocols that have dramatically improved the data acquisition throughput. Nowadays, the collection of about 50 tomograms per day without any user interference has become feasible. It has recently been shown that subtomogram averaging holds the potential to resolve secondary structure. Whether this will be achieved for nuclear pores in the near future depends on several conditions: (1) Structure determination of membrane embedded, intact NPCs relies on relatively thick specimen, such as nuclear envelopes. It is thus dependent on energy-filtered electron detectors that reduce the contribution of inelastic electron scattering events observed on the camera. Next generation direct electron detectors coupled to energy filters have recently become available and should be explored for structure determination of NPCs. They promise to increase the signal to noise ratio of the primary data at least 2-fold. (2) The data acquisition throughput might be even
An important caveat about tomographic structures is that it is not entirely clear which parts of the nuclear pore are averaged out during the structure determination process because they are flexible. On the other hand, even non-nucleoporins might contribute to observed electron optical density. One might assume that intrinsically disordered domains (IDPs) are averaged out while structured scaffold and anchoring domains are retained. However, scaffold Nups might be also flexible to quite some degree, while IDPs might generate structured elements. The latter is nicely illustrated by the fact that the dynein light chain-interacting (DID) domain of yNup159 that is classified as an IDP, forms a highly structured rod when interacting with dynein.15

In order to interpret the tomographically observed electron optical density, it will be of utmost importance to further characterize isolated subcomplexes by single particle EM. Unfortunately, this has turned out challenging for Nups. Because of their membrane-associated nature, Nup subcomplexes often assume a preferred orientation on EM grids and may even disintegrate during the preparation of grids. To the best of our knowledge, no cryoEM structures of subcomplexes have been reported thus far. Although negative staining EM has revealed very important insights into subcomplex structures, one needs to bear in mind that the available negative staining structures might have been flattened along one spatial direction during the staining procedure. This problem can potentially be addressed by exposing the sample to less favorable conditions for surface adherence during EM grid preparation and the application of thicker embedded staining as we have done for the hNup107 subcomplex.7 A promising alternative is the use of functionalized surfaces4,15 or even membranes that were successfully used for elucidating structures of coated vesicles using tomographic approaches.10

Another important aspect to take into account when studying the structure of nuclear pores and their subcomplexes is that they are quite heavily post-transnationally modified. Prominent post-translational modifications (PTMs) of Nups are phosphorylation,17-20 O-glycosylation,21-23 N-glycosylation,24 sumoylation,25 ubiquitination,26 and acetylation.27 Advances in the mass spectrometry hardware, such as, e.g., the increased scanning speed of Orbitrap instruments or the introduction of electron dissociation transfer (ETD) fragmentation together with specific peptide enrichment strategies have permitted the large-scale identification of PTMs. Recent large scale studies have discovered a considerable number of PTM sites of Nups, see, e.g., references 20,23,25,27. To the best of our knowledge, the regulatory function of at least some of PTMs abundant in Nups, e.g., acetylation, remain largely understudied. Interestingly, some of these, e.g., O-glycosylation and phosphorylation, have a high likelihood to co-occur at neighboring residues and to co-evolve28 and might even interplay at the same residue23 such as, e.g., acetylation and sumoylation.27 These PTMs modulate various functional aspects of the nucleocytoplasmic transport system. For example, it is known that vertebrate FG Nups are modified by O-linked glycosylation,22 and this might regulate the vertebrate NPC permeability barrier.29 Phosphorylation of Nup98 is a key step during mitotic disassembly of NPC50 and high-throughput studies have demonstrated that various Nups are heavily phosphorylated during mitosis.20 One might thus assume that phosphorylation is important to regulate inter-subcomplex interfaces of nucleoporins and that the hNup107 subcomplex is potentially a key player in the events of open mitosis. In line with this hypothesis, we have recently shown that phosphorylation sites map in potential mitotically regulated inter-subcomplex interfaces of the overall Y subcomplex. Phosphorylation sites within ELYS, Nup133 and Nup96 localize into these essential inter-subcomplex contact sites, further underlining the hypothesis that such areas are prime targets for investigating the disassembly and re-assembly mechanism of temporal cell cycle intermediates.7 We mapped several phosphorylation sites in the pertinent sections of Nup160 that might be functionally relevant for NPC assembly. Also the distal N-terminus of Nup133 is heavily phosphorylated, likely to control the head-to-tail interaction of the hNup107 subcomplex in this flexible region. The N-terminal part of Nup96, which appears highly flexible in isolated subcomplexes, is heavily phosphorylated suggesting that it might assume a different conformation in assembled NPCs, where it localizes into the vertex interface.7 These regions are prime for regulatory activity.

Confirmed in our work are 48 previously not annotated serine/threonine phosphorylation sites within the hNup107 subcomplex. By combining affinity isolation of mitotic Nups with the enrichment of phosphorylated peptides,
we were able to identify a considerably larger number of phospho-peptides within the hNup107 subcomplex than aforementioned high-throughput studies that solely relied on a phospho-capturing strategy. This example demonstrates that similar biochemical strategies might be employed for various PTMs and subcomplexes in order to more comprehensively map the PTM landscape of Nups across different biological states. Such data will be crucial to understand how PTMs modulate NPC structure. Furthermore, it will be important to conduct such studies in vertebrate and mammalian cells since PTMs seem to play important roles in biological processes that are absent in yeast, such as mitotic nuclear envelope breakdown and embryonic development. Notably, phosphorylation sites are also observed in yeast Nups and likely have other important regulatory roles. 

Advancing toward a high-resolution structure of the vertebrate NPC clearly requires progress on various different experimental fronts other than discussed above. For example, some domain folds and a large number of protein interfaces await high-resolution structural analysis. Separate targeting of poorly or non-conserved nucleoporins from lower and higher eukaryotes seems necessary. The fact that some nucleoporin domains are obviously engaged in multiple distinct protein interactions thereby imposes an additional layer of complexity. A better understanding of protein-lipid interactions as well as the contribution of IDPs to the scaffold architecture is needed as well. Finally, joint efforts of various laboratories would be the appropriate way to tackle the data integration challenge, since the weight of different types of data is often difficult to judge individually.

Disclosure of Potential Conflicts of Interest
No potential conflict of interest was disclosed.

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