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Review

One Ring to Rule them All? Structural and Functional Diversity in the Nuclear Pore Complex

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The nuclear pore complex (NPC) is the massive protein assembly that regulates the transport of macromolecules between the nucleus and the cytoplasm. Recent breakthroughs have provided major insights into the structure of the NPC in different eukaryotes, revealing a previously unsuspected diversity of NPC architectures. In parallel, the NPC has been shown to be a key player in regulating essential nuclear processes such as chromatin organization, gene expression, and DNA repair. However, our knowledge of the NPC structure has not been able to address the molecular mechanisms underlying its regulatory roles. We discuss potential explanations, including the coexistence of alternative NPC architectures with specific functional roles.

Highlights

The NPC is a large protein assembly that regulates macromolecular transport between the nucleus and the cytoplasm, and acts as a regulatory platform for many other essential nuclear processes.

NPC structural characterization has been challenging, but recent technical and methodological advances are advancing our understanding of NPC architecture.

Structural analyses of the NPC in different organisms revealed that, although there is a common bauplan, a significant degree of variability is observed in the peripheral modules that build these NPCs.

The multiple regulatory roles shown for the NPC suggest an even greater degree of NPC architectural diversity that has not yet been unveiled.

The NPC Regulates Trafficking In and Out of the Nucleus

The nucleus is undoubtedly the quintessential eukaryotic organelle. The nucleus of a cell is defined by the presence of a double lipid membrane, termed the **nuclear envelope** (NE; see Glossary), that surrounds and encloses the chromatin. The flux of genetic information as defined by the central dogma of molecular biology (DNA makes RNA makes protein) is divided into two main steps, transcription and translation, that are physically segregated by the NE. To overcome the NE barrier and ensure fast, constant, and regulated exchange of macromolecules between the nucleus (where transcription takes place) and the cytoplasm (where translation happens), eukaryotic cells have developed a remarkable molecular machine, a massive protein complex called the **nuclear pore complex** (NPC). The NPC is a large (~100 nm wide and ~40 nm high) eightfold symmetrical assembly composed of more than 550 copies of ~30 different proteins termed **nucleoporins** (Nups) [1–3]. The NPC core scaffold coats the NE membrane, shaping and stabilizing it to form a central channel of ~40–60 nm that traverses the NE and acts as the sole communication hub between the inside of the nucleus and the cytoplasm. The central channel of the NPC is filled by a mixture of transport factors and their cargoes, together with intrinsically disordered Nup domains rich in **phenylalanine-glycine (FG) repeats** [4,5], that collectively make up the so-called central transporter [6]; although the transporter and mechanism of transport are not our main focus, we address some interesting recent insights in Box 1. Instead, we summarize our knowledge of the NPC structure across organisms, and discuss the implications of those findings in the context of our understanding of the multiple roles that the NPC plays as a platform for regulating nuclear processes.

The NPC Is a Key Regulatory Platform for Nuclear Processes

Although the paramount function of the NPC is to mediate transport between the nucleus and the cytoplasm, research in recent years is also highlighting the importance of the NPC as a regulatory platform. The nuclear side of the NE can be envisaged as a surface in which NPCs are prominent reference points where molecular machineries can be recruited, anchored, and coordinated. This

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Box 1. The Nuclear Pore Complex (NPC) Central Transporter

The central transporter is a mixture of proteins which form the permeability barrier that regulates transport and maintains the selectivity of the NPC. A significant part of the central transporter is formed by FG-repeat nucleoporin (Nup) domains (main text) [122]. Only cargo proteins containing specific signals can be recognized by transport factors that then, through specific interactions with these FG repeats, traverse the NPC with their cargoes [123–125]. Approximately one-third of all Nups contain these FG regions, and their close-packed anchoring along the walls of the central channel forms a high-density **polymer brush**. Although the biophysical nature and molecular mechanism of nuclear transport remains a topic of heated debate, there is now some consensus that FG repeats function as a highly dynamic and fluid 'phase' *in vivo* [124,126,127] because FG repeats are intrinsically disordered regions that are either weakly cohesive or non-cohesive, and form a highly mobile tethered solution in the central transporter that excludes non-specific macromolecules while allowing the rapid passage of transport factors. Crucially, the bulk of this selective 'phase' is not made of FG-repeat regions; instead, roughly three-quarters is actually composed of a heterogeneous mixture of transport factors and their cognate cargos [1,128].

Cryo-EM studies have provided few hints about the organization for the central transporter, but this is not surprising because the central transporter is highly heterogeneous, extraordinarily dynamic, and is in significant part disordered. However, a few studies did resolve the central transporter as an hourglass-shaped density that is connected through thin bridges to the inner-ring region in the NPC equator [1,6,129]. The position of these bridges coincides with the predicted emanating points for the Nic96 complex FG domains (Nsp1, Nup57, and Nup49; Figure 1), strongly suggesting that the bridges are formed by these FG regions emanating as plumes from the inner ring to form the transporter [1]. This observation constitutes the first hint about the organization of the central transporter, but to unravel its full dynamic organization and mechanism will likely require numerous alternative and complementary approaches. For example, groundbreaking advances have been made by the use of **atomic force microscopy**, a method that was used to image the shape and stiffness of the *X. laevis* central transporter [130], and, when used in its high-speed mode, to observe the time-resolved dynamics of its components [131].

dual role for NPCs as hubs of both communication and regulation was suggested by Günter Blobel [7] and has been expanded upon by a multitude of subsequent studies (reviewed in detail in [8–11]). NPCs have been shown to organize chromatin and regulate gene expression by partially overlapping mechanisms that can be broadly grouped into: (i) modulation of chromatin state and architecture [12–14], (ii) post-translational modification of chromatin-associated proteins [15–18] or Nups themselves [19], and (iii) tethering of specific transcription factors to control gene positioning by recruitment of their cognate DNA-binding sites [20–22]. In addition to these roles, the NPC is also involved in maintaining genome integrity; upon the appearance of particular DNA lesions, DNA damage response components are actively recruited to peripheral regions of the NPC for repair of the DNA insults [23–25].

Structure of the NPC: One Size Does Not Fit All

Despite a remarkable amount of effort, we are still far from a clear mechanistic understanding of most of the NPC functional roles summarized above. Because 'structure determines function' in biology, one of the key pieces of information that was missing to solve this puzzle was a complete and comprehensive description of the structure of the NPC at the molecular level. Once we know the arrangement of each Nup and subcomplex, we should be able to start to make sense of the accumulated functional data by placing these in a proper structural context. However, the sheer size of the NPC, its complexity, and the presence of a significant amount of disordered proteins in its composition, have made the structural characterization of the NPC a formidable challenge. Only the combination of groundbreaking technical and methodological advances in different areas, including biochemistry, proteomics, **integrative methods**, crystallography, and **cryo-electron microscopy** (cryo-EM), have finally allowed the field to tackle this problem and gain a higher-resolution view of the structure of the NPC in a variety of organisms (Figure 1).

To build an NPC, most Nups assemble into biochemically stable subcomplexes that in combination form eight identical protomer units termed 'spokes'. These spokes were first described by EM [26], and, although the terminology now is established, we may need to consider more accurate

Glossary

Atomic force microscopy: a technique that enables imaging the surface of a sample and analysis of its mechanical properties by the use of a sharp tip ~10–20 nm in diameter attached to a cantilever.
Cryo-electron microscopy (Cryo-EM): an imaging technique that analyzes the shape and dimensions of a frozen-hydrated sample using transmission EM under cryogenic conditions.
Cryo-electron tomography (Cryo-ET): a cryo-EM method in which the samples are imaged as they are tilted at defined angles relative to the electron beam, to obtain a series of 2D images that are then computationally combined into a 3D reconstruction of the sample.
Focused ion beam (FIB): a microscopy technique based on the use of a focused beam of ions to ablate layers of material in a biological sample, carving it into a thin layer with nanometer precision; it is usually applied to frozen-hydrated specimens and combined with cryo-EM and tomographic methods.
Integrative method: a strategy for determining the structure of biological systems based on data produced by multiple experimental and theoretical methods that are combined into a single, coherent structural calculation; the combination of several complementary methods to solve a structure is also called a hybrid approach.
Membrane-binding motifs (MBMs): short amino acid sequences that directly interact with the NE membrane; they are usually amphipathic α-helices that insert their hydrophobic region into one face of the membrane lipid bilayer.
Nuclear envelope (NE): a double lipid membrane that is contiguous with the endoplasmic reticulum and that surrounds, delimits, and shapes the nucleus in eukaryotic cells.
Nuclear pore complex (NPC): a large protein complex that creates a channel through the NE and mediates transport of macromolecules between the nucleus and the cytoplasm.
Nucleoporin (Nup): a protein constituent of the NPC, defined as a protein that spends most of its life in the NPC and forms a stable part of its structure.
Phenylalanine-glycine (FG) repeats: intrinsically disordered Nup domains that are rich in FG SLiMs separated by very hydrophilic spacer residues.

descriptions as more is revealed about NPC fine structure. Each of the eight spokes is attached to the NE membrane, and connect radially to each other to form concentric rings: the outer, inner, and membrane rings. Each ring runs parallel to the equatorial plane of the NPC: a single membrane ring protrudes towards the NE lumen at the point of junction between the outer and inner NE membranes, two inner rings delineate the central channel, and two outer rings sandwich the inner rings and stabilize the NE membrane curvature as it enters the NPC. The outer rings also serve as anchoring platforms for asymmetric Nups and cofactors that form the cytoplasmic mRNA export machinery and the nuclear basket [1,27–29]. All these major modules of the NPC are connected by an extensive network of flexible connectors [30] – intrinsically disordered Nup domains containing **short linear motifs** (SLiMs, more detailed explanation is given below) – that act as flexible cables that tie together the more rigid modules to generate a strong but flexible assembly [1].

Polymer brush: a mass of polymer chains tethered to a solid substrate that have a density higher than the polymer radius of gyration.
Short linear motifs (SLiMs): intrinsically disordered domains within some Nups that contain one or more short interaction sequences; these interaction sequences establish specific, but not extensive, interactions with folded domains of large Nups and NPC subcomplexes.
Y-complex: an NPC module that forms the outer rings; it is formed by 6–9 Nups and adopts a characteristic Y-shape.

Several models for the structure of the NPC in different organisms are now available [1,28,29,31,32]. Together, these have revealed two key insights. The first is that the inner rings of all analyzed NPCs share an overall conserved arrangement. However, the second insight is that different organisms show significant variability in the more peripheral regions of the NPC; a diversity of NPC architectures is thus generated by gain or loss of components, as well as different combinations and stoichiometries of conserved building modules. As a consequence, we can now safely state that there is no such a thing as 'the' structure of the NPC, instead

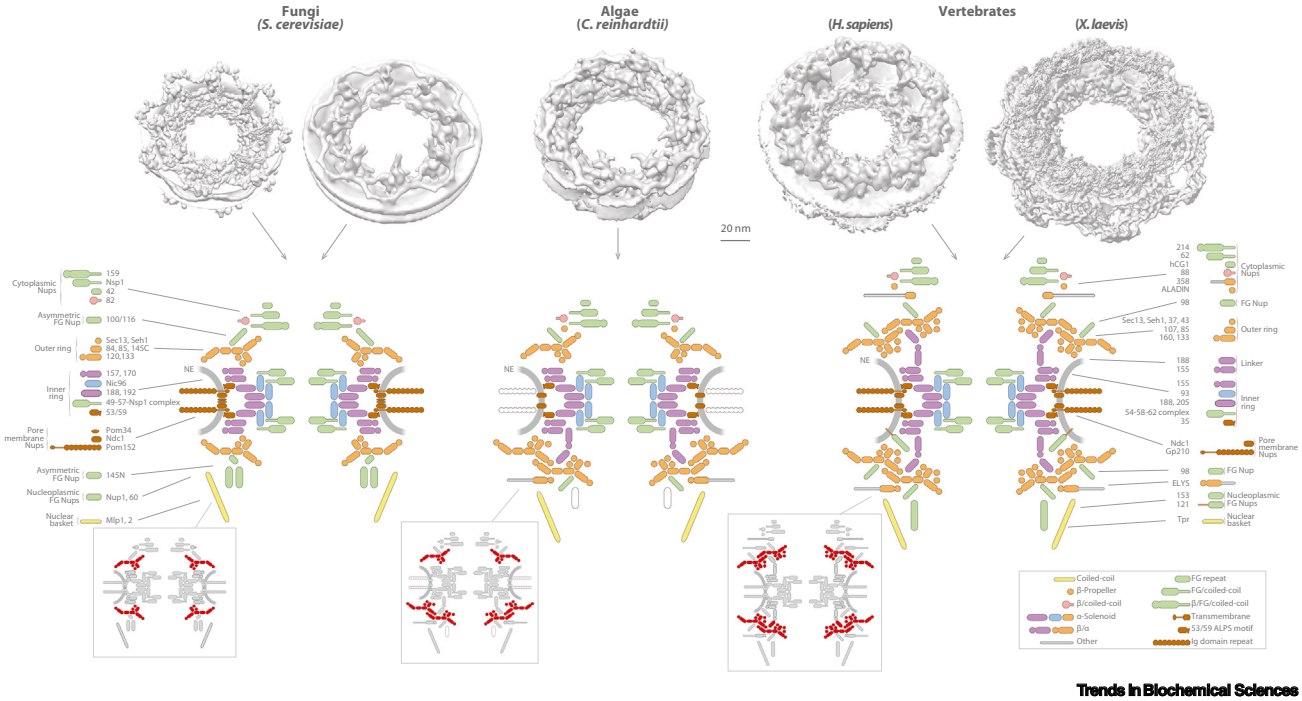

Figure 1. Structural Diversity of Nuclear Pore Complexes (NPCs) across Eukaryotes. (Top row) NPC structures for the indicated organisms solved by integrative methods (left, *Saccharomyces cerevisiae* PDBDEV_00000010-12 [1]), FIB-cryo-ET (right, *S. cerevisiae* EMD-10198 [31], and *Chlamydomonas reinhardtii* EMD-4355 [32]), cryo-ET (*Homo sapiens* EMD-3103 [47]), or cryo-ET and single-particle analysis (*Xenopus laevis,* image kindly shared by Gaoxingyu Huang and Yigong Xi [57,80]). (Middle row) Diagrams reflecting the known composition and arrangement of the different nucleoporins (Nups) and NPC modules in *S. cerevisiae* (left), *C. reindhardtii* (middle), and *H. sapiens* (right). The identity of each Nup is shown to the left in *S. cerevisiae* or to the right of the *H. sapiens* diagram. Key: empty, non-colored shapes indicate a non-identified component; gray arches represent the nuclear envelope (NE). (Bottom row) NPC diagrams showing the outer ring components in red to highlight their alternative arrangement in the different organisms. (Bottom right corner) Box depicting the protein folds and domains found in Nups; β/α indicates a β-propeller followed by an α-solenoid. Abbreviations: cryo-ET, cryo-electron tomography; FIB, focused ion beam.

there is a common structural bauplan from which a – so far uncharacterized – variety of structures arose through evolution to adapt to the biological needs and constraints of each organism [1,33–37]. Using the model organism *Saccharomyces* (baker's yeast) as our initial reference for the NPC, we summarize what is currently known about the structure of the different NPC modules, starting from the conserved core scaffold and then working our way outwards to the more variable peripheral regions, discussing their alternative architectures and their possible biological relevance.

The Inner Ring

The inner rings form the structural heart of the NPC, and as such represents its most conserved module. The inner rings are roughly symmetric with respect to the equator of the NPC, with two superposed symmetric and laterally offset rings, one located on the cytoplasmic side and one on the nuclear side. Equatorially, the inner ring extends from the NE membrane all the way to the central channel, serving both as a framework to shape and stabilize the membrane and as the anchor point for much of the rest of the NPC core scaffold, as well as for most of the mass of FG Nups that form the central transporter (Figure 1).

The main Nup components of the inner-ring scaffold are relatively large proteins (>90 kDa) that are formed by either α-helical solenoid domains or a specific combination of N-terminal β-propeller and C-terminal α-helical solenoid [38]. The α-helical solenoid and β-propeller/α-solenoid signature, that is also found in the outer-ring Nups (see following text) [39], is only found in components of other membrane-coating complexes – such as clathrin/adaptin, COPI, and COPII – and tethering complexes such as IFT, SEA, and HOPS/CORVET. Such architectural similarities between molecular machineries devoted to molding membranes led to the proposal of a common evolutionary origin for the NPC and these coating complexes in an ancestral protocoatomer complex, from which they have inherited and retained structural and mechanistic signatures [27,38–40].

The closest inner-ring components to the NE membrane are a double pair of paralogous proteins, the β-propeller/α-solenoid Nups – Nup157 and Nup170 – and the flexible connector containing Nup53 and Nup59. All these Nups contain **membrane-binding motifs** (MBMs) [41–43], that appear to anchor them to the membrane around the point of insertion of the transmembrane domains of Pom152, the main component of the membrane ring. Nup157 and Nup170 are oriented perpendicular to the equatorial plane of the NPC, whereas Nup53 and Nup59 extend from the membrane into the central channel, connecting with other major Nups of the inner ring and also serving as bridges between adjacent spokes [1,28,29] that are also connected through relatively small interaction surfaces between opposing Nup170 β-propellers [1]. Further from the NE membrane, and adjacent to the surfaces of Nup157 and Nup170 facing the central channel, four copies of Nic96 form a diagonally oriented column across the spoke that serves as the keystone of the NPC [1,28,29], holding the large α-solenoids of Nup192 and Nup188, bracing Nup170 and Nup157, and orienting four coiled-coil trimeric Nsp1–Nup57–Nup49 complexes [44,45] (Figure 1). These coiled-coils enclose the central transport channel and serve as projection points for the FG repeat domains of Nsp1–Nup57–Nup49 that represent ~4 MDa worth of mass in the central transporter. The flexible connector domains of Nup53/Nup59 and Nup145N/Nup116/Nup100 thread through the whole inner ring, interconnecting its components and tying it to the outer rings and mRNA export machineries on both the nuclear and cytoplasmic sides of the NPC [1].

Although the overall architecture of the inner ring was found to be relatively conserved in the organisms studied so far, clear variability has been observed in the degree of compaction, overall dimensions, and manner of connection to the outer rings [1,28,29,31,32]. One of the most

obvious differences is the presence of additional copies of a β-propeller/α-solenoid Nup157/Nup170 ortholog, termed Nup155, that form upward-facing pillars, running roughly perpendicular to the NPC equatorial plane, and connecting the inner rings to the outer rings in the vertebrate NPC [46,47]. These connection pillars are missing in the *Saccharomyces cerevisiae* NPC [1,31] and appear to be present only on the nuclear side of the algae *Chlamydomonas reinhardtii* NPC [32]. Although the functional reason for the presence of these pillars is not clear, one can speculate that their role could be related to the difference in the height of the NE between these organisms (vertebrate ~40 nm; algae and yeast ~25 nm) and by the presence of staggered double rings in both vertebrate outer rings and in the algae nuclear outer ring (see following text) [32,46,47], which might require additional spacers to span this extra width and maintain NPC integrity.

Another difference is that the diameter of the human [48], algae [32], and yeast [31] NPCs appears significantly wider when analyzed by *in situ* **cryo-electron tomography** (ET) (central channel ~60 nm) than in isolated vertebrate or yeast NPCs (~40 nm) [1,47]. Apparently, this difference in diameter does not seem to involve major rearrangements of the architectures of the inner ring and spokes [31,32,48]. Such ranges in diameters have been observed in vertebrate NPCs using other methods [49,50], collectively leading to the speculation that these different conformations might reflect a continuum of dynamic dilation/constriction that the NPCs could adopt in response to physiological events [51,52]. Indeed, a groundbreaking study recently showed that transmission of mechanical force from the cytoskeleton to the NE results in stretching of the NPCs that changes their permeability and allows nuclear import of a mechanosensitive transcriptional activator, YAP [53]. This malleability of the diameter of the NPC central channel may be facilitated because the lateral contacts between spokes at the inner ring are limited to a few, small interaction surfaces, as discussed previously. Such thin connections should allow some degree of sliding and opening of the spokes to adapt to mechanical stress [54], thus facilitating the passage of large cargoes or coordinating responses to extracellular stimuli by changes in the NPC permeability barrier [53].

The Membrane Ring

The membrane ring runs parallel to the equatorial plane of the NPC and is formed by integral membrane Nups with varying numbers of transmembrane regions [27] (Figure 1). Until recently, no indication of its arrangement was available from cryo-EM analyses [46,47]. However, studies in fungi [1,55,56] and *Xenopus* oocytes [57] have provided a first glance at the surprisingly diverse arrangement of this part of the NPC. In yeast, the main component of the membrane ring, Pom152, forms homodimeric interactions through their elongated pearl-on-string luminal domains that generate arches connecting adjacent spokes within the NE lumen [1,55]. The wider part of these arches coincides with the boundaries between spokes, delineating what may be the transport pathway for integral membrane proteins [1]. The remaining components of the membrane ring (Pom34 and Ndc1) are structurally less well defined, although they seem to help to establish multiple connections between the NE membrane and the major Nups of the inner ring [1,58].

A recent analysis performed in *Xenopus laevis* oocyte NEs identified a structural feature that most likely corresponds to the vertebrate membrane ring [57] (Figure 1). Unexpectedly, it is not a diaphanous feature, and is instead a bulky density formed by multiple copies of an elongated protomer (reminiscent of the Pom152 luminal domain shape [55,56]) that arrange to form a two-winged symmetric subunit in each spoke. Wing-to-wing connections are established between spokes to form the so called 'bumper domains', that were suggested to help to cushion neighboring NPCs when they get close to each other in the highly crowded oocyte NE (~60 NPCs

per square micron) [57]. The most likely scenario is that the elongated protomers are mainly formed by the luminal domain of vertebrate Nup210, although the presence of other proteins could not be excluded. Nup210/Gp210 is the largest component of the vertebrate membrane ring, and appears to be structurally homologous to yeast Pom152; although it is not present in some metazoan cell lines [59,60], it has been shown to be a key regulator of gene expression and cell differentiation processes [61]. Thus, confirming that Nup210 is the protomer that generates the membrane ring in *X. laevis* oocytes will be vital to start placing these functional data into a structural context and to arrive at a mechanistic understanding of how changes in NPC composition can define cellular fate.

The NPC Outer Rings and Associated Peripheral Machineries

The conserved building block of the NPC outer rings is the so-called **Y-complex**, that owes its name to its characteristic elongated Y-shape [62,63]. Based on the organisms characterized so far, this complex has a conserved heterohexameric core that delineates the Y, and a variable number of additional components that add up to nine members in the vertebrate complex [46,64–67]. Most of the conserved components are either β-propeller/α solenoid proteins (Nup133, Nup120 [68–71]) or COPII-like α-solenoid Nups (Nup145C, Nup85, and Nup84 [72–74]), adding to the overwhelming evidence for a common evolutionary origin between the NPC and membrane-coating complexes in an ancestral protocoatomer [39] (reviewed in detail in [34,75]). The Y-complex contacts the NE membrane through MBMs located at the tips of the complex, within the β-propellers of Nup120 and Nup133 [1,42,46,68,76,77], and it arranges in a head-to-tail fashion to form the outer rings on both sides of the NPC [27,64,71,77].

However, the number of Y-complex copies in each outer ring has been shown to vary from organism to organism (Figure 1). In the human NPC, two staggered rings of eight Y-complexes each form both cytoplasmic and nuclear outer rings, adding up to 32 copies of the complex per NPC [46,78], whereas in yeast only one octameric ring per side (16 Y-complexes per NPC) has been observed [1,27]. Remarkably, an algae cryo-EM NPC map indicates an intermediate composition, with a yeast-like cytoplasmic single-ring arrangement and a vertebrate-like nuclear double staggered ring (24 Y-complexes per NPC) [32]. An even more complex arrangement has been recently suggested for the organization of the outer rings of the fission yeast *Schizosaccharomyces pombe* [79]; using immunoelectron and fluorescence microscopy methods, Asakawa *et al.* observed that components of the Y-complex are asymmetrically distributed between the nuclear and cytoplasmic outer rings, resulting in an arrangement of fragmented Y-complexes that would strongly deviate from the canonical view of how the outer rings are organized [79].

Whenever a double Y-complex ring has been identified, eight β-propeller/α-solenoid Nup155 pillars have been shown to connect it to the inner ring [32,46,47], and additional components have been proposed to stabilize the arrangement. First, the vertebrate-specific Nup358/RanBP2 is present in the cytoplasmic outer ring, where it forms a 'clamp' around the stem of the double Y-complex ring [47,80]. This Nup358 clamp helps to stabilize the arrangement because gene silencing of Nup358 leads to disassembly of the outer Y-complex ring [47]. Second, question-mark-shaped densities, tentatively assigned to Nup188 based on crosslinking and mass spectrometry data [28,29,46], are present in each spoke connecting the double Y-complex rings on both sides of the NPC. However, a higher-resolution cryo-EM map of the *X. laevis* cytoplasmic outer ring indicated instead that these are formed by two copies of Nup205 [80]. It is thus an open question whether this is a conserved or a differential feature between nuclear and cytoplasmic spokes, whether the number of outer rings is diagnostic for

the path of NPC evolution between organisms, as proposed [33], or whether it is a further feature that can be regulated to meet differential demands on NPC functionality.

The modules that control RNA remodeling and export in the NPC, the cytoplasmic export platform and the nuclear basket, are anchored to the outer rings, although they also make connections with the inner rings [1]. Mechanistically, the export of RNA is largely distinct from that of other cargoes. First, RNAs are packaged into export-competent ribonucleoproteins that dock to the nuclear basket. These are then chaperoned across the NPC by specific transport factors. Finally, they are remodeled and released by the export platform on the cytoplasmic face of the NPC [81–83]. Both the basket and the export platform seem to have a major attachment point in the short arm and hub of the most internal Y-complex [1,84], where they likely establish contacts with the Y-complex backbone and through SLiMs located in flexible connectors, as shown in the case of a thermophilic fungus [85]. The core scaffold of the cytoplasmic RNA export platform is the Nup82 complex that is formed by a complex arrangement of heterotrimeric coiled-coil bundle subunits [84,86]. Variability in the components of the Nup82 complex between yeast and vertebrates suggest that the detailed architecture of the complex may vary between species and may be a somewhat malleable structure, but cryo-EM maps agree in positioning the Nup82 complex as a hook-like structure that protrudes into the central channel of the NPC [1,31,32,46,80,84], where it recruits the RNA helicase Dbp5 and associated cofactors to disassemble the messenger ribonucleoproteins in the last step of nuclear export [82,87].

Although it is known that its major component is the large coiled-coil protein Tpr in vertebrates [88,89] and its homologs Mlp1/Mlp2 in *Saccharomyces* [90,91], the arrangement and orientation of the nuclear basket are still poorly defined, probably owing to the intrinsic flexibility and potential heterogeneity of Tpr/Mlp and its other components, including the observation that not all NPCs seem to possess a nuclear basket [90,92,93]. *In situ* **focused ion beam** and cryo-ET analysis of *Chlamydomonas* cells revealed filament-like structures attached to the nuclear side of the NPC [94] that would be reminiscent of our traditional picture of the nuclear basket, but their nature has not been verified and it is not clear whether such an arrangement is present in other type of cells. Instead, we still rely on much older work to give us some idea concerning the organization of these structures, such as high-resolution scanning EM images of both yeast and vertebrates which showed that the nuclear basket is composed of eight filaments attached to the nucleoplasmic ring to conjoin with a distal basket ring [95,96].

Flexible Connectors

It has been estimated that ~60% of the NPC mass comes from the relatively large α-solenoid/β-propeller-containing Nups and coiled-coil bundles that form the core scaffold of the outer and inner rings, and these components generate most of the observed density in the published NPC cryo-EM structures [1,3]. However, there are much less noticeable NPC elements that nevertheless perform the crucial role of tying together all these large NPC modules. First identified in Ed Hurt's laboratory [30], they have been called SLiMs or flexible connectors, and they are extended, intrinsically disordered domains within particular Nups that contain one or more short interaction sequences. These sequences establish specific but not extensive interactions with the folded domains of large Nups and subcomplexes [97]. Taken together, the evidence indicates that the flexible connector-containing Nups form an extensive network of 'cables' that extend vertically, from the cytoplasmic face of the NPC through the inner ring and all the way to the nuclear side, and horizontally, extending from the NE membrane, traversing the inner ring, and connecting to the central transporter, towards which some of them project their associated FG regions, generating a flexible but stress-resilient structure [1,30,85,98].

It is relatively common to find FG domains that associate with flexible connectors within Nups, and it has been suggested that both domains may have a common evolutionary origin [99], and that they could act synergistically to modulate their interaction with folded Nups and karyopherins during NPC biogenesis and to maintain the stability of the mature NPC [100,101]. Several of the SLiMs have been crystallized in interactions with their Nup partners [29], and some have been shown to only interact with assembled NPC subcomplexes, mainly those formed by coiled-coil bundles [30,44,85], strengthening the idea that they might be important for coordination during NPC biogenesis. In summary, the flexible connectors perform an analogous role to that of the suspender cables in a bridge, tying together the major modules to provide flexibility and strength to the structure.

Can We Understand the Regulatory Roles of the NPC in the Light of Its Structure?

Although in this short review we can only provide the briefest synopsis, we hope to have shown the reader that in the past few years the field has come a long way in its efforts to dissect the architecture of the NPC. Nevertheless, the variabilities that have been observed clearly indicate that we may still be far from grasping the real structural and functional diversity of the NPC across eukaryotes, and even within individual cells. The picture that is emerging is that of a relatively conserved core to which peripheral modules can be plugged in and out, potentially combining into a multitude of possibilities, similar to the popular Lego toy suite. Such a 'plug and play' character for its peripheral regions might explain some of the regulatory mechanisms discussed previously for the NPC because changes in those modules would imply changes in their associated interactomes and functional purposes. However, the situation appears to be more nuanced because recent studies are challenging the idea that the NPC regulatory roles are solely related to its more variable and less conserved outer peripheral regions, and instead show that components buried in the core structure of the NPC, that are allegedly relatively inaccessible to interaction, play key roles in gene expression regulation.

The most remarkable cases are the metazoan inner ring components Nup155 and Nup93, and their fungal homologs Nup170 and Nic96, respectively (Figure 1). Although both proteins are buried within the structure of the inner ring, they have been shown to have conserved roles in the recruitment of heterochromatin to the NPC. In *Saccharomyces*, Nup170 was shown to recruit the silencing factor Sir4 and promote transcriptional repression of subtelomeric regions at the NPC [102]; its metazoan homolog, Nup155, was also shown to interact with the histone deacetylase HDAC4 [103] and to be required for chromatin association to the NPC in an RNA interference screen looking for factors required for chromatin detachment in meiotic *Drosophila* oocytes [12]. Similarly, a pair of recent studies demonstrated that *Drosophila* Nup93 [104] and its fission yeast homolog Npp106 [105] are physically associated with heterochromatin and are involved in gene silencing. Human Nup93 has also been shown to participate in binding superenhancers [106] as well as in tethering and repressing the *HOXA* gene cluster [16], suggesting a highly conserved role for Nup93 in the recruitment and silencing of chromatin at the NPC. Together, these studies strongly indicate that two Nups buried at the core of the NPC spoke, where the available structures show that they act as true structural keystones and anchor points for many other Nups, are nevertheless able to establish physical interactions with bulky silencing protein complexes and heterochromatin domains within the NPC.

How could these two apparently conflicting possibilities be reconciled into a coherent structure/function model for the NPC? Paradoxically, there is plenty of evidence suggesting that other Nups and NPC modules may restrict heterochromatin access to the inner-ring Nups or even serve to recruit actively transcribed genes. A negative regulatory loop in which Nup62 can repress the observed Nup155 chromatin-tethering activity has been described in *Drosophila* [12], and the

presence of the nuclear basket seems to be required for the exclusion of heterochromatin and for maintaining an open chromatin architecture at NPCs, as suggested by studies in fission yeast [105] and virus-infected human cells [107–109]. Recent reports describe the association of euchromatin and active genes with outer-ring complex components in the NPC [21,104], supporting previous studies that showed recruitment of actively transcribed genes [61,110] and a role for the NPC as a key platform for transcriptional memory and the tethering of inducible genes [111,112]. We are thus facing apparently contradictory evidence indicating that, on the one hand, within a relatively compact, inaccessible NPC structure, several of its inner components are nevertheless directly involved in recruiting heterochromatin and promoting gene silencing, whereas, on the other hand, peripheral components recruit euchromatin, to prevent access of heterochromatin to the NPC, and thus support expression of active genes.

Based on our knowledge of NPC biology, we speculate on several non-mutually exclusive scenarios that could help to explain this apparent conundrum: first, the remarkable flexibility of the NPC core scaffold (discussed in the preceding text) might facilitate openings in the structure that would expose particular components to interaction with cofactors and other assemblies; second, Nups could have 'out-of-the-NPC' functions because several NPC components have been shown to perform intranuclear regulatory roles independently of their association with the NPC [17,113–117], although the vast majority of inner-ring Nups in a cell are devoted to building NPCs [2], and they perform most of their regulatory functions as part of the mature NPC [17,104,106]; finally, a third explanation that would fit with the available evidence would be the coexistence of different types of NPCs or Nup assemblies that have alternative architectures and differential functionalities within the same cell types.

This type of scenario arose, for example, from the work of Lapetina *et al.* [118] where, during dissection of the Nup170 and silencing factors interaction network in *Saccharomyces*, they identified what they called the Snup, an assembly formed by core Nups (mainly members of the inner and outer rings) that is physically distinct from a mature NPC and does not contain FG, flexible-connector, or nuclear-basket Nups. The authors suggest that the Snup, and not the mature NPC, is responsible for interacting with silencing factors such as Sir4 and drives the recruitment of subtelomeric chromatin [118]. Although more detailed work will be necessary to investigate the exact nature of the Snup, this study, together with those previously mentioned, raises the interesting possibility that alternative NPC architectures with distinct functional roles might coexist in the same cell. Similarly to the architectural variability observed between divergent eukaryotes (Figure 1), an unidentified diversity of NPC architectures might be present within a single cell or be assembled in response to particular physiological cues. Examples are already present in the literature, where NPCs lacking a nuclear basket were detected around the nucleolus in *Saccharomyces* cells [92,93], distinct NPC architectures were found in the *Tetrahymena* somatic macronucleus and germline micronucleus [36], and changes in Nup stoichiometry were described across human cell types [3,119,120].

Concluding Remarks

Far from the traditional, somewhat monolithic picture of a ring that we have seen for decades in textbooks, technical and methodological advances in the structural characterization of the NPC are revealing a beautifully intricate architecture with a significant degree of both compositional and conformational flexibility [1,28,29,31,32,57,80]. To explain the malleable character of the functional architecture of the NPC, we have previously used the analogy of a suspension bridge [1]: both the NPC and a suspension bridge are structures that create a stable passageway for a large and continuous flow of traffic that passes through them at all times; both are formed by firm anchors to a substrate (NE or bedrock, respectively) and comprise relatively rigid modules that

Outstanding Questions

How is the NPC eightfold symmetry defined, and what are the structural determinants that define the number of modules that form each NPC?

What are the molecular determinants that define the oligomerization state of an NPC outer rings? What is the functional reason behind a single or double outer-ring arrangement?

How do Nups, that do not have any obvious DNA-binding motifs, interact with chromatin for its regulation? If it is through adaptor proteins, what are they and how is their interaction with the NPC modulated?

Do all NPCs transport the same type of cargo? Are there specialized NPCs devoted to organizing scaffolds for gene regulation?

Do all NPCs have a membrane ring? How does the composition of the vertebrate NPC membrane ring define its ability to regulate gene expression and cell-fate determination?

Does the Snup complex represent a different type of mature NPC, an NPC biogenesis precursor, or a completely different assembly?

are connected by hinges and a network of cables tying all the modules together. This type of architecture produces a flexible but resilient assembly that could accommodate many types of stresses without suffering damage to its integrity. We could now even expand this analogy because, like bridges, the NPC seems to have been shaped into diverse incarnations and variations that accommodate the differing needs of a variety of organisms.

How the observed alternative NPC architectures relate to the different biologies of these organisms remains an open question, as is the question of how these alternative NPC architectures were shaped during eukaryotic evolution [33,34] (see Outstanding Questions). Moreover, recent advances in the functional characterization of the NPC are helping to underscore its key role as a nuclear regulatory platform that operates independently from its primary role as a regulator of transport between the nucleus and the cytoplasm. The NPC is directly involved in regulating a large variety of pathways and physiological processes, including chromatin architecture or transcriptional control and memory [9,10,121]. It is thus hard to imagine how a protein assembly could support such a variety of functional roles without undergoing significant changes in both its composition and/or structure. Similarly to the diverse NPC architectures identified across organisms, we expect that a so far unidentified variety of NPCs might exist even within single tissues and cell types. If confirmed, this exciting possibility would open vast new avenues of research that would require the development of novel approaches and methodologies to explore the diverse and dynamic nature of NPCs.

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Declaration of Interests

The authors declare no conflicts of interest.

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