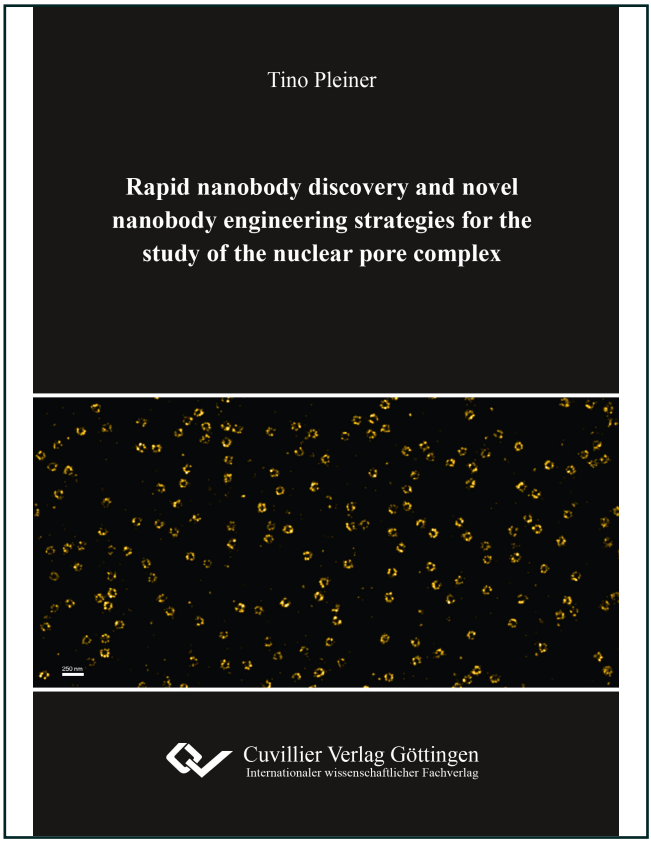
Tino Pleiner (Autor)

Rapid nanobody discovery and novel nanobody engineering strategies for the study of the nuclear pore complex



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2 Introduction

2.1 The vertebrate nuclear pore complex (NPC)

A double membrane-bound nucleus is the defining feature of all eukaryotic cells. The nuclear envelope encloses the cell's genetic information and strictly regulates access to it from the surrounding cytoplasm. The advantages of this compartmentalization are manifold. The spatial separation of transcription (nucleus) and translation (cytoplasm) is the basis for the very high fidelity of gene expression and effectively prevents the production of potentially toxic protein fragments from incompletely or incorrectly processed mRNAs. It also enabled alternative mRNA processing mechanisms to evolve that yield different protein products from a single gene thereby increasing the complexity of eukaryotic cells. Furthermore, the regulatory control over access to the genome allowed a temporal-spatial fine-tuning of gene expression e.g. under different environmental conditions or differentiation states.

This physical division of eukaryotic cells necessitates an exchange of macromolecules between the two compartments. Nucleocytoplasmic transport proceeds through circular openings that fenestrate the nuclear envelope (Callan and Tomlin, 1950; Feldherr, 1962). These nuclear pores result from the fusion of the outer and inner nuclear membrane. While the outer nuclear membrane is continuous with the endoplasmic reticulum, access to the inner nuclear membrane is restricted and it thus has a distinct protein composition.

Giant multi-protein assemblies reside in nuclear pores (Gall, 1954; Watson, 1959) and constitute a physical permeability barrier that prevents an intermixing of nuclear and cytoplasmic contents. Under the electron microscope these so-called nuclear pore complexes (NPCs) (Watson, 1959), show an octagonal symmetry along the transport axis (Gall, 1967) (Figure 2.1). The cytosolic side of NPCs shows eight filamentous protrusions, whereas the nuclear side carries a filamentous basket-like structure.

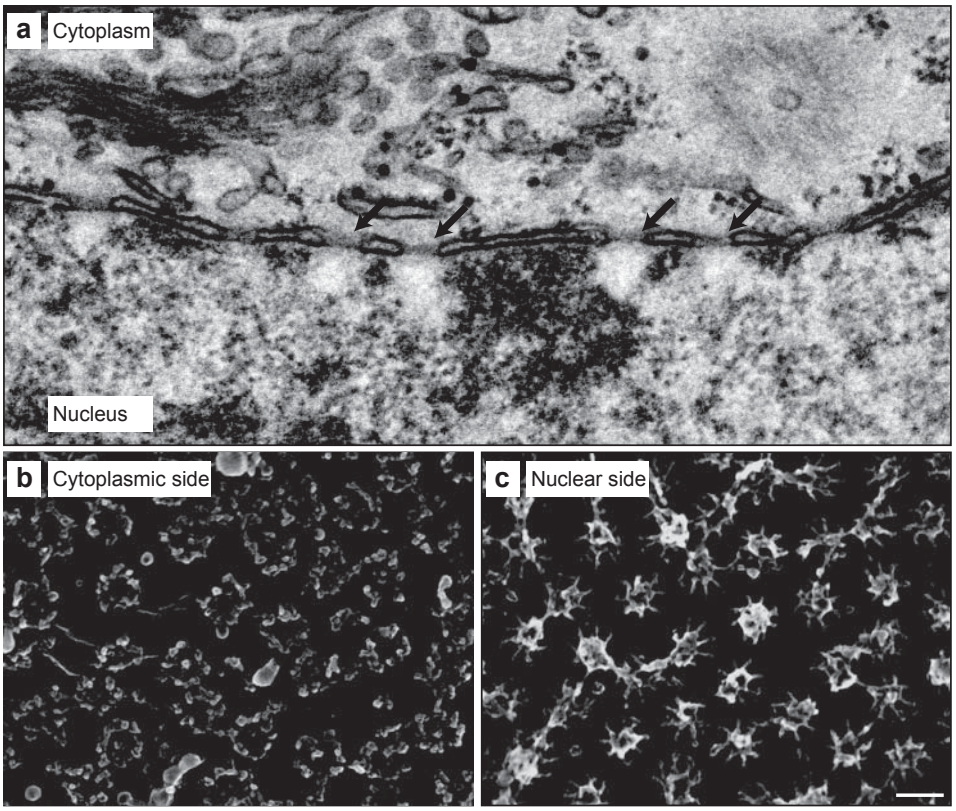

Figure 2.1. Nuclear pore complexes are embedded in the envelope of cell nuclei.
(**a**) Transmission electron micrograph of a HeLa cell nuclear envelope cross-section. Black arrows indicate regions of the nuclear envelope that contain nuclear pores at sites where the outer and inner nuclear membrane are fused. The nuclear side of the pores shows distinct exclusion zones that correspond to the nuclear basket region. (**b**) Scanning electron microscopy (SEM) image of a *Xenopus* oocyte nuclear envelope from the cytosolic side. The octagonal nuclear pore complexes display unconnected filamentous protrusions. (**c**) SEM image of a *Xenopus* oocyte nuclear envelope from the nuclear side. Filamentous basket-like structures emanate from the nuclear pore complexes. All images were kindly provided by Dr. Volker Cordes (MPI for Biophysical Chemistry, Göttingen).

2.1.1 Nucleocytoplasmic transport

Nucleocytoplasmic exchange across the nuclear envelope can occur in two modes – passive and facilitated (reviewed in Görlich and Kutay, 1999). Simple passive diffusion is efficient only for proteins less than 20-30 kDa in size and 4-5 nm in diameter (Mohr et al., 2009). Above this size limit passive diffusion is very slow. Facilitated nuclear transport is a carrier-mediated process and very efficient even for very large proteins and protein complexes. This mode of transport allows directional import and export of proteins and requires the cargo molecule to possess a specific signature or transport signal that can recruit so-called nuclear transport receptors (NTRs). The first nuclear transport signal to be identified is the classical nuclear localization signal (cNLS) that

confers nuclear import and consists of a stretch of basic amino acids (Dingwall et al., 1982; Kalderon et al., 1984; Lanford and Butel, 1984).

The cNLS is recognized by the import adaptor protein Importin-α (Adam and Adam, 1994; Görlich et al., 1994), which mediates binding to the major NTR of the Importin (Imp) family, Impβ (Adam and Adam, 1994; Chi et al., 1995; Görlich et al., 1995; Imamoto et al., 1995). The Impβ•Impα•cargo complex then traverses the NPC. Nuclear pore passage is per se bidirectional, energy-independent and requires interaction of Impβ with Phe-Gly (FG) repeat-containing proteins of the NPC. Binding of the small GTPase Ran (Melchior et al., 1993; Moore and Blobel, 1993) in its GTP-bound form (RanGTP) to Impβ on the nuclear side of the NPC releases the cargo and Impα (Görlich et al., 1995; Rexach and Blobel, 1995). The Impβ•RanGTP complex then traverses back to the cytoplasmic side of the NPC where disassembly is catalyzed by RanBP1 and rendered irreversible by Ran GTPase activating protein (RanGAP)-stimulated GTP hydrolysis of Ran (Bischoff and Görlich, 1997). This frees Impβ to participate in another round of import and leaves RanGDP in the cytoplasm. Impα also needs to be recycled back to the cytoplasm. A specific export-mediating NTR Exportin (Xpo)-2 (or CAS) performs this task (Kutay et al., 1997).

Exportins bind their cargo together with RanGTP in the nucleus (Stade et al., 1997; Fornerod et al., 1997; Kutay et al., 1997). Assembly of the trimeric Exportin•RanGTP•cargo complex is cooperative and leads to its translocation through the NPC. On the cytoplasmic side, export complex disassembly is triggered by RanBP1 and RanGAP, leading to cargo and RanGDP dissociation. The Exportin can then cycle back to the nuclear side to transport another cargo. The major Exportin of the cell, CRM1, has a very broad cargo range and recognizes classical nuclear export signals composed of multiple spaced hydrophobic residues (Fornerod et al., 1997; Güttler et al., 2010; Kırlı et al., 2015).

Both import and export processes constantly shuttle Ran to the cytoplasm where it is converted to its GDP-bound form (RanGDP). RanGDP is shuttled back to the nucleus by a dedicated transport receptor, called Ntf2 (Ribbeck et al., 1998). Importantly, Ntf2 does not require RanGTP for cargo unloading and thus achieves a net transport of Ran. In the nucleus the chromatin-bound guanine nucleotide exchange factor Rcc1 (Bischoff and Ponstingl, 1991) converts RanGDP to RanGTP. The asymmetric localization of

proteins regulating Ran's nucleotide-bound state ensures the establishment of a steep RanGTP gradient across the nuclear envelope that confers directionality to all transport events and renders them irreversible (Görlich et al., 1996b; Görlich et al., 1996a; Izaurralde et al., 1997).

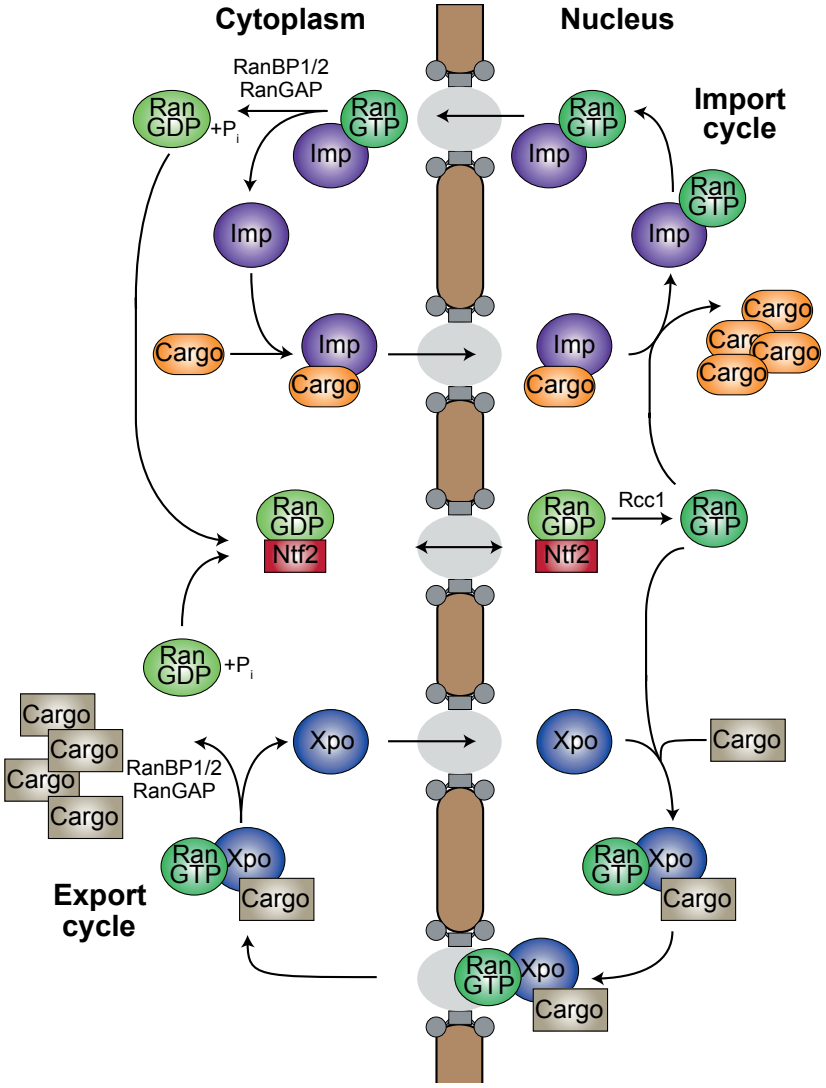

Figure 2.2. Schematic overview of facilitated nucleocytoplasmic transport.
Facilitated nuclear transport is divided into import and export processes. Importins (Imp) bind their substrates in the cytoplasm, where RanGTP levels are low. Imp•cargo complexes then traverse the NPC and are dissociated at the nuclear side by RanGTP binding to the Imp. This triggers a conformational change and results in cargo release. The Imp•RanGTP complex cycles back to the cytoplasm where GTP hydrolysis by Ran is triggered by the NPC-bound proteins RanGAP, RanBP1 and RanBP2. This frees the Imp to participate in another round of import. An Export complex assembles in the nucleus upon RanGTP and cargo binding to an Exportin (Xpo). The trimeric complex then traverses the NPC to the cytoplasmic side where GTP hydrolysis leads to its disassembly. The Xpo cycles back to the nucleus where it mediates another round of export. In order to prevent the dissipation of the RanGTP gradient (high concentration in nucleus, low concentration in cytoplasm) RanGDP is transported back to the nucleus by a specific NTR called Ntf2. Transport is per se bidirectional and energy-independent. The RanGTP gradient confers directionality and GTP hydrolysis renders the transport processes irreversible. This figure is adapted from Görlich and Kutay, 1999.

2.1.2 Structure of the nuclear pore complex

The structural organization and composition of the NPC is important for understanding its essential function in nucleocytoplasmic transport as a highly selective permeability barrier. Therefore, it has been the subject of intensive investigation. Due its enormous size of around 120 MDa in higher eukaryotes (Reichelt et al., 1990; Ori et al., 2013), structural analysis of the NPC is challenging. Cryo-electron tomography emerged as a suitable technique to study the overall architecture of NPCs (Hinshaw et al., 1992; Bui et al., 2013; Eibauer et al., 2015; von Appen et al., 2015). The NPC scaffold spans around 114 nm in width and 80 nm in height (Figure 2.3). It consists of three ring-like structures that are interconnected and individually anchored to the membrane. Due to a pseudo-twofold symmetry relative to the nuclear envelope plane, the cytoplasmic and nuclear rings appear very similar in architecture. The inner ring shows a distinct structure and encloses the narrowest constriction (~40 nm) of the central transport channel.

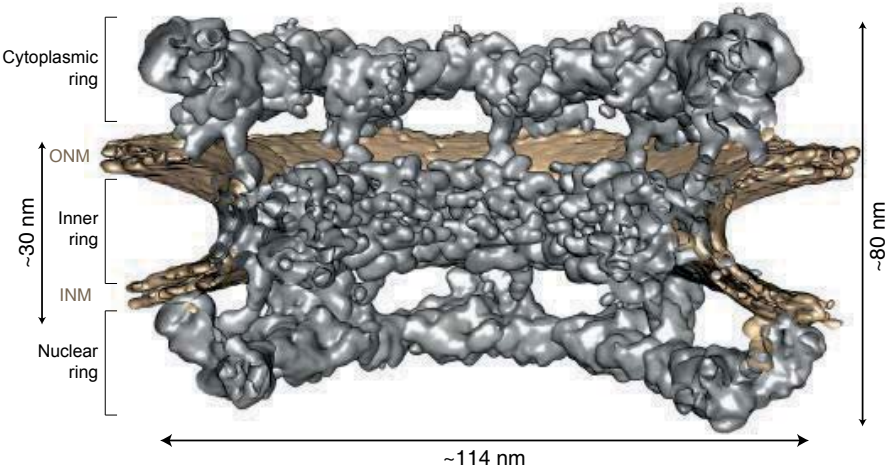

Figure 2.3. Structure of the human nuclear pore complex scaffold.
The nuclear pore complex (NPC) is a large multiprotein complex that resides in pore-like openings of the nuclear envelope where outer (ONM) and inner nuclear membrane (INM) are fused. Due to its large size of around 120 MDa cryo-electron tomography (ET) has proven to be the method of choice for structural analysis of the NPC. This image shows a recent cryo-ET reconstruction of the human NPC at 23 Å resolution (prepared from EMD-3103 with UCSF Chimera; von Appen et al., 2015). A cutaway view of the electron density is shown. The major scaffold of the NPC (dark grey) (excluding cytoplasmic filaments and nuclear basket) spans around 114 x 80 nm and has an eight-fold rotational symmetry axis (8 asymmetric units). The NPC is divided into three interconnected ring-like structures (cytoplasmic, inner and nuclear ring). All rings show distinct contact regions with the membrane (brown).

The eightfold-rotational symmetry of the NPC suggested it to be quite a modular assembly. Indeed, only around 30 different proteins (Cronshaw et al., 2002), called

nucleoporins (Nups), form a NPC (Figure 2.4; Figure 2.5). Nups assemble into subcomplexes that can be found as stable building blocks throughout the cell cycle. The subcomplexes in turn form the entire NPC and likely exist in a copy number that is a multiple of 8 (e.g. 16 or 32-fold) (Figure 2.5) (Ori et al., 2013). Nups can be broadly classified into transmembrane Nups, scaffold Nups and Phe-Gly (FG) repeat containing Nups, although this distinction is not absolute and some Nups seem to have dual roles (Fischer et al., 2015). The NPC scaffold is anchored to the pore membrane via transmembrane Nups, whereas the scaffold itself positions FG Nups in the central transport channel. The non-globular FG repeat domains of FG Nups are thought to interact with each other to form a three-dimensional meshwork that constitutes the permeability barrier (Ribbeck and Görlich, 2001; Frey et al., 2006; Frey and Görlich, 2007; Hülsmann et al., 2012).

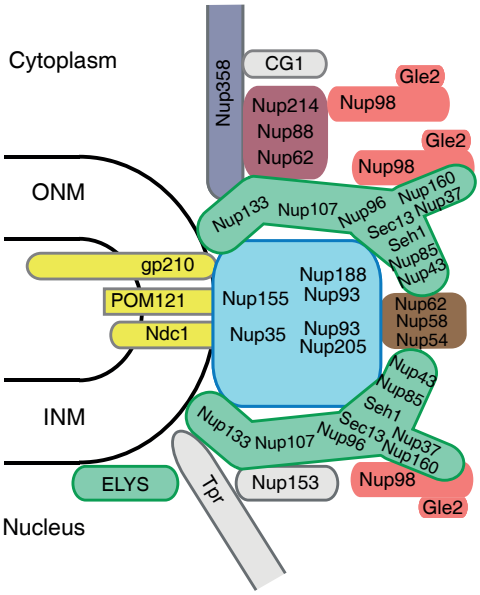

Figure 2.4. Composition of the NPC.
Schematic representation of the subcomplex organization and relative localization of Nups within an asymmetric unit of the eightfold rotational symmetric vertebrate NPC. The nuclear and cytoplasmic rings of the structural NPC scaffold are mainly composed of the Nup107 or Y-shaped complex (green). The central inner ring is composed of the Nup93-containing subcomplex (blue). The scaffold is bound to the nuclear envelope via transmembrane Nups (yellow) and further anchors FG-repeat nucleoporins (e.g. Nup98 (red) and the Nup62•Nup58•Nup54 complex (brown)) within the central channel, where they form the permeability barrier. Distinct Nups are present at the cytosolic and nuclear side. The Nup214•Nup88•Nup62 complex is anchored at the cytoplasmic Nup107 complex ring. Nup358/RanBP2 forms the cytoplasmic filaments. Nup153 is a specific binding partner of the Nup107 complex at the nuclear ring. The large Nup TPR forms the nuclear basket. ONM/INM = outer and inner nuclear membrane.

2.1.3 Composition of the NPC

The relative arrangement of nucleoporins within the NPC was primarily studied with classical immunogold labeling electron microscopy (Rout et al., 2000; Krull et al., 2004) or with indirect immunofluorescence. Conflicting localization data for many Nups, as a result of these low-resolution approaches, however, make it still quite hard to get a coherent understanding of the molecular architecture of the NPC.

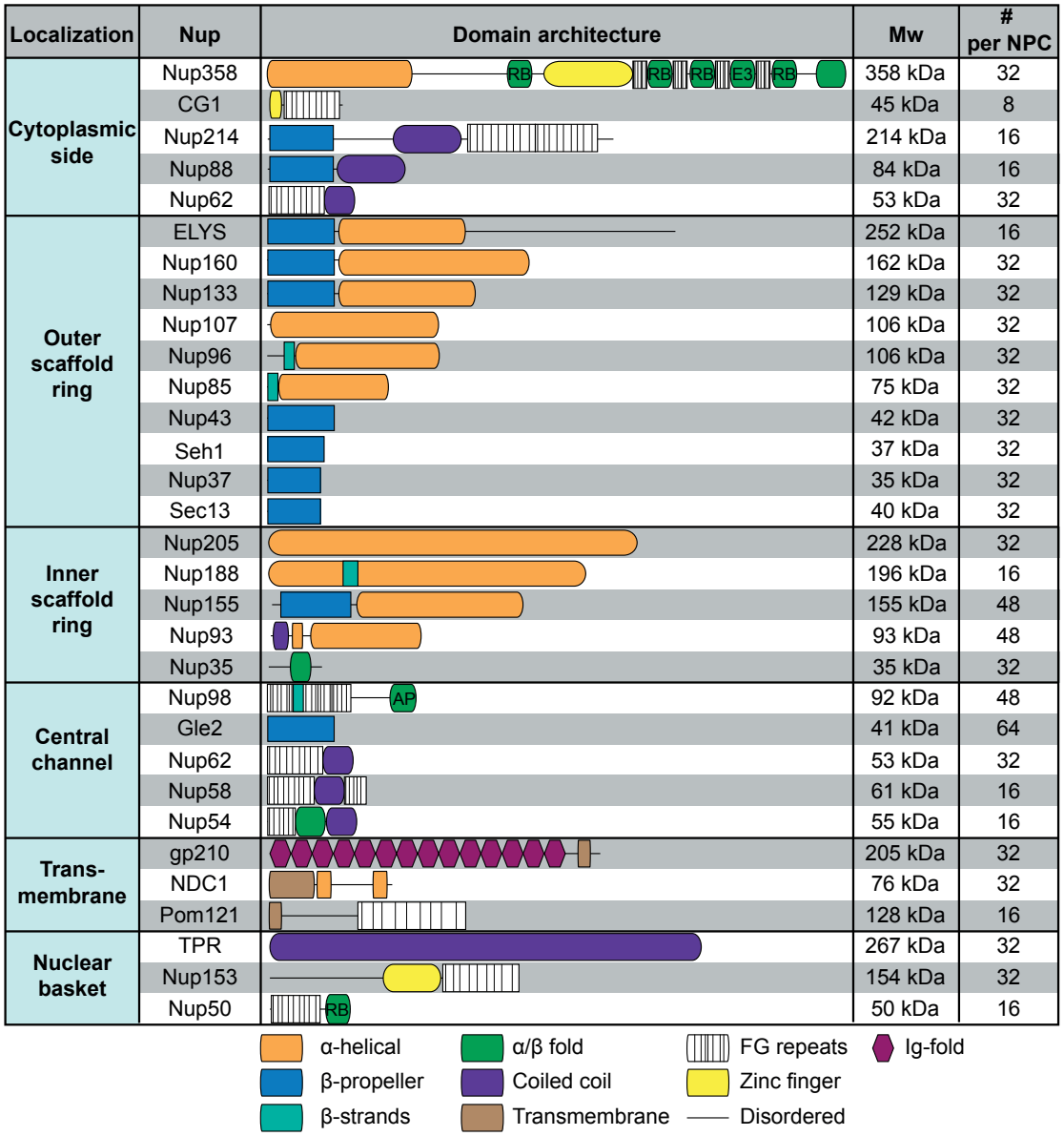

Figure 2.5. Nucleoporin inventory of human NPCs and their domain architecture.
Around 30 different proteins are present in vertebrate NPCs in multiple copies. The approximate copy numbers of all Nups (# per NPC) were recently estimated based on mass spectrometry data (Ori et al., 2013). Two fold types are predominantly found in Nups: α-helical repeats and β-propellers. Many Nups contain incomplete β-propellers that are complemented in trans by other Nups that insert a single β-blade (e.g. Nup98•Gle2, Nup96•Sec13, Nup85•Seh1). Ten Nups possess non-globular Phe-Gly (FG)-repeat domains that bind nuclear transport receptors and participate in the formation of the permeability barrier. The trimeric Nup62•Nup58•Nup54 complex is formed by extensive coiled coil interactions that also contribute to NPC anchorage via Nup93. The Nup214•Nup88•Nup62 complex also involves a coiled-coil interface. RB = Ran-binding domain; E3 = E3 SUMO Ligase domain; AP = autoproteolytic domain. This figure is adapted from Brohawn et al., 2009; as well as from Knockenhauer and Schwartz, 2016.

Cytoplasmic side

The cytoplasmic filaments of the NPC were shown to be dispensable for nuclear transport in an *in vitro* system (Walther et al., 2002). They are likely formed by the very large nucleoporin RanBP2/Nup358 (Wu et al., 1995; Yokoyama et al., 1995) that lacks

a homologue in lower eukaryotes. RanBP2 has four Ran-binding domains, possesses FG repeats and forms a complex with sumoylated RanGAP (Mahajan et al., 1997). In fact, RanBP2 is itself an E3-SUMO Ligase (Pichler et al., 2002). *In vivo* studies suggested that RanBP2 functions as a platform for efficient nuclear import complex formation (Hutten et al., 2008; Hutten et al., 2009). Recently, an unanticipated role of RanBP2 in organizing the structural arrangement of the cytoplasmic ring was suggested (von Appen et al., 2015).

The Nup214•Nup88•Nup62 complex is a trimeric FG repeat-containing coiled-coil complex (Macaulay et al., 1995; Bastos et al., 1997; Belgareh et al., 1998) that functions as a docking site for CRM1 export complexes (Fornerod et al., 1997; Bernad et al., 2006) and forms a protrusion from the cytoplasmic ring towards the central channel (Bui et al., 2013; Gaik et al., 2015).

It is still unclear how exactly RanBP2 and the Nup214•Nup88•Nup62 complex are anchored to the NPC scaffold.

Outer scaffold rings

A large conserved nucleoporin subcomplex called Nup107-160 or Y-complex (Siniossoglou et al., 1996; Siniossoglou et al., 2000; Vasu et al., 2001) is essential for NPC assembly (Harel et al., 2003; Walther et al., 2003) and forms the cytoplasmic and nuclear rings of the NPC scaffold (Bui et al., 2013). It is the best-characterized NPC subcomplex and consists of ten proteins in higher eukaryotes. Its structural arrangement within the NPC has been intensely studied and multiple different models were proposed (Alber et al., 2007; Hsia et al., 2007; Brohawn et al., 2008). Data derived from various techniques suggested a head-to-tail arrangement of the Y-complex (Alber et al., 2007; Kampmann et al., 2011; Szymborska et al., 2013). Recently, a cryo-EM reconstruction of the human Y-complex could be fitted into a cryo-electron tomogram of the human NPC revealing a surprising double head-to-tail ring arrangement in both cytoplasmic and nuclear ring (16 copies per ring, 32 in total) (Bui et al., 2013; von Appen et al., 2015). Many Nups or Nup fragments of the Y-complex were crystallized individually before and recently also in complex allowing the construction of atomic 3D models of the entire complex (Stuwe et al., 2015b; Kelley et al., 2015).

There is still a debate on whether there is a single or a double head-to-tail ring (Kelley et al., 2015) and whether this arrangement is identical in the cytoplasmic and nuclear rings (Eibauer et al., 2015). Furthermore, species and cell-type-specific differences in NPC structure and composition might exist and are still poorly understood.

Inner scaffold ring

The nucleoporins Nup93, Nup188, Nup205, Nup155 and Nup35 constitute the inner ring of the NPC scaffold, whose architecture is less well understood (Vollmer and Antonin, 2014). Nup93 is essential for NPC assembly and occurs in two distinct subcomplexes with the two large paralogous proteins Nup188 and Nup205 (Grandi et al., 1997; Miller et al., 2000; Theerthagiri et al., 2010; Sachdev et al., 2012). The binding of Nup188 and Nup205 to Nup93 is mutually exclusive and both are dispensable for NPC assembly (Theerthagiri et al., 2010; Sachdev et al., 2012). The yeast Nup93 homologue Nic96 was crystallized and shows a J-like α-helical fold (Jeudy and Schwartz, 2007; Schrader et al., 2008). Fungal Nup188 and Nup205 assume characteristic S-shaped structures in cryo-EM and bear some resemblance to nuclear transport receptors (Amlacher et al., 2011; Flemming et al., 2012; Andersen et al., 2013). All three proteins were shown to bind FG repeats and this could contribute to a tight sealing of the permeability barrier on the NPC scaffold (Schrader et al., 2008; Andersen et al., 2013).

Nup35 and Nup155 are both essential for NPC assembly (Franz et al., 2005; Hawryluk-Gara et al., 2008), interact with each other and undergo a complicated interaction network with Nup93 and Nup205 of the inner ring (Hawryluk-Gara et al., 2005), as well as with NDC1 and Pom121 at the nuclear pore membrane (Mansfeld et al., 2006; Mitchell et al., 2010; Eisenhardt et al., 2014). Additionally, both proteins were suggested to directly contact membranes (Vollmer et al., 2012; von Appen et al., 2015). They therefore have a major role in membrane anchorage of the NPC scaffold.

Recently, various inner ring scaffold complexes were reconstituted *in vitro* with well-behaved proteins from the thermophilic fungus *Chaetomium thermophilum* (Amlacher et al., 2011; Fischer et al., 2015). The three-dimensional organization of these very flexible complexes within the NPC remains a challenging puzzle for future research.

Central Channel

The central channel is delineated by FG repeat nucleoporins that form the permeability barrier (Labokha et al., 2013; Schmidt and Görlich, 2016). The structural organization of the permeability barrier is debated, although biochemical data suggest a gel-like FG meshwork (Hülsmann et al., 2012). Therefore, future research needs to obtain information about the exact copy number, 3D localization and configuration of FG Nups to arrive at a mechanistic understanding of nucleocytoplasmic transport.

A prominent FG-repeat Nup complex is the trimeric coiled-coil Nup62•Nup58•Nup54 complex. It is anchored to the inner ring scaffold via coiled-coil interactions with the N-terminus of Nup93. Its stoichiometry and structural organization was the subject of a series of speculative models that were not supported by data (Melcák et al., 2007; Solmaz et al., 2011; Solmaz et al., 2013; Sharma et al., 2015; Koh and Blobel, 2015). Only recently, its 1:1:1 stoichiometry was confirmed by biochemical and structural data (Ulrich et al., 2014; Chug et al., 2015; Stuwe et al., 2015). Interestingly, the kinked and elongated complex could only be crystallized with bound antibodies (camelid nanobody by Chug et al., 2015; synthetic antibody by Stuwe et al., 2015). Recent functional analyses illustrated that NPCs with a functional, although slightly compromised, permeability barrier can form in the absence of the Nup62•Nup58•Nup54 complex (Hülsmann et al., 2012).

Nup98 is the only GLFG-repeat Nup of higher eukaryotes (Radu et al., 1995) and has three orthologues in yeast (Wente et al., 1992; Wente and Blobel, 1994). Nup98 is produced from a larger Nup98-Nup96 precursor protein via autoproteolytic cleavage or via alternative splicing from the precursor mRNA (Fontoura et al., 1999). The absolute C-terminus of Nup98 anchors it at the NPC via interaction with Nup96 and Nup88 in a mutually exclusive manner (Hodel et al., 2002; Griffis et al., 2003; Yoshida et al., 2011; Stuwe et al., 2012). Likely the ‘unstructured region’ and the FG-repeat domain of Nup98 contribute to its NPC anchorage (Griffis et al., 2003; Yoshida et al., 2011; Hülsmann et al., 2012; Fischer et al., 2015). A short β-strand segment, called GLEBS domain, is located within the FG domain of Nup98 and mediates interaction with the essential mRNA export factor Gle2 (Bailer et al., 1998; Pritchard et al., 1999). Importantly, the cohesive FG-repeat domain of Nup98 was found to be essential for formation of the permeability barrier (Hülsmann et al., 2012). Moreover, the