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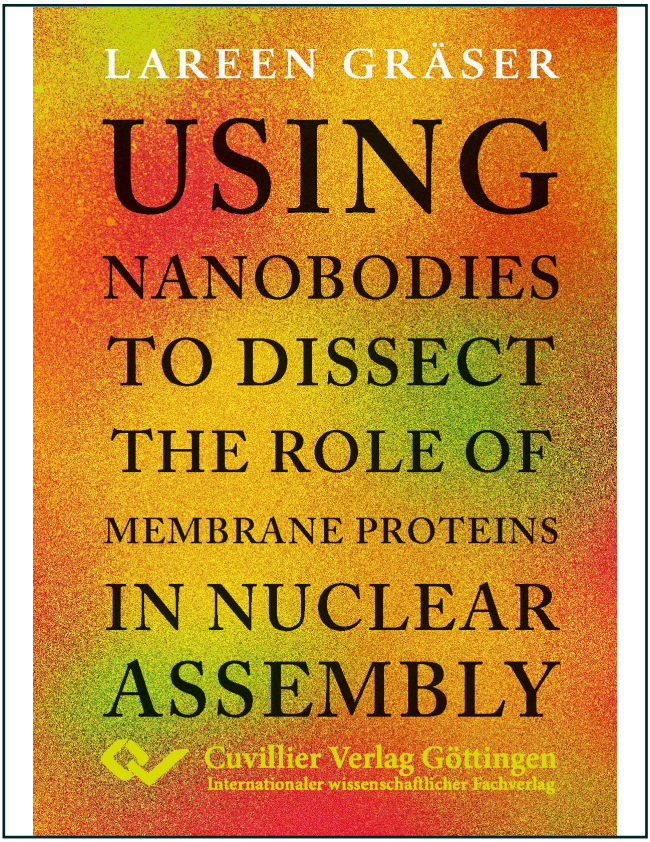
Using nanobodies to dissect the role of membrane proteins in nuclear assembly



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Introduction

The focus of this study is on membrane proteins located at the endoplasmic reticulum (ER) and the connected nuclear envelope (NE). Therefore, the following sections will discuss the involved compartments and the corresponding components to ultimately move towards the investigated proteins. The second part of the introduction provides an overview of the characteristics and applications of single domain antibodies (nanobodies) as they were used as a tool to study these proteins.

1.1 The architecture of the endoplasmic reticulum

In eukaryotic cells, cellular components are spatially segregated by single or double lipid-layered membranes. This compartmentalization establishes physical boundaries for biological processes, generating a specific microenvironment within each compartment (e.g. Cavalier-Smith, 1988). The cytoplasm houses different membrane-bound structures. Each of them specializes in different metabolic processes and thus contains an individual protein composition, both inside and on the respective membranes.

The ER is the largest of these compartments and serves many different functions such as calcium storage, protein synthesis and folding, or lipid metabolism. The peripheral ER consists of interconnected tubules as well as flattened sheets that span throughout the cytoplasm (Porter, 1953; Veratti, 1961). Due to their distinct functions, the peripheral ER can be further divided into rough and smooth ER. The membrane protein composition of the two types of ER is similar but not equal. The rough ER is, among other things, responsible for the translocation of proteins, which is why proteins contributing to this function are enriched in the rough ER (e.g. Kreibich et al., 1978; Vogel et al., 1990). The ER is a highly dynamic

structure formed by processes such as elongation and shortening of tubules or tubule branching (Lee and Chen, 1988). These tubules are maintained and shaped by various different proteins such as reticulon proteins or DP1 (e.g. Voeltz et al., 2006; Hu et al., 2008). The ER membranes enclose a lumen that is fully continuous, also with that of another part of the ER, the nuclear envelope.

1.2 The nuclear envelope

The NE is a specialized part of the ER and surrounds the nucleoplasm, including the chromatin. It is composed of a membrane sheet of two lipid bilayers, further classified as outer nuclear membrane (ONM) and inner nuclear membrane (INM). These membranes fuse at various sites to embed nuclear pore complexes (NPCs) that are critical for bidirectional exchange between the nucleus and the cytoplasm (e.g. Gerace and Burke, 1988; Watson, 1955). In higher eukaryotes, the NE is mechanically supported by a meshwork of intermediate filament proteins underlying the INM, A-type, and B-type lamin, which were first described in electron microscopic studies by Patrizi and Poger (1967). Furthermore, lamin and lamina-associated proteins (LAPs) anchor chromatin to the NE through lamina-associated domains (LADs) (e.g. Wilson and Foisner, 2010).

Overall, the NE harbors at least 60 NE-specific membrane proteins, most of them likely in the INM (e.g. Schirmer et al., 2003). However, several ONM proteins have been identified that perform functions such as anchoring the nucleus through the association of nuclear lamina with the cytoskeletal system. ONM proteins involved in this function contain a KASH domain at their C-terminus, which contributes to interactions with SUN-domain proteins (part of the INM) within the perinuclear space (PNC) (Padmakumar et al., 2005; Wilhelmsen et al., 2006).

Numerous integral membrane proteins reside in the INM, also called nuclear envelope transmembrane proteins (NETs), most of which remain poorly characterized. One of the better studied complexes in the INM is the LINC (Linker of Nucleoskeleton and Cytoskeleton) complex. As indicated by its name, the LINC complex constitutes a connection between the nucleoplasm and the cytoplasm. On the INM site, it is composed of the SUN domain-containing proteins SUN1 and SUN2, which extend to the PNC where they tether ONM-spanning nesprin proteins (Crisp et al., 2006; Sosa et al., 2012).

LAP2, emerin, and MAN1 form another family of NET proteins and are defined by their

shared LEM domain (∼ 40 amino acids) (Lin et al., 2000). They have various functions and binding partners, but all three proteins commonly bind to the barrier-to-autointegration factor (BAF), which is associated with chromatin (e.g. Furukawa, 1999; Shumaker et al., 2001; Bengtsson and Wilson, 2004).

1.3 Nuclear Pore Complex

NPCs are embedded in the NE and regarded as the hallmark of eukaryotic cells. They ensure bidirectional exchange by forming a channel that functions as a selective physical barrier for nucleocytoplasmic transport of macromolecules (e.g. Gerace and Burke, 1988). The NPC was investigated using electron microscopy (EM), in isolated nuclei from *Xenopus laevis* oocytes, as well as in isolated nuclei from *Triturus* oocytes (e.g. Callan and Tomlin, 1950; Gall, 1954). The initial hypothesis postulated a circular shape of the NPC until Franke (1966) and Gall (1967) revealed a ring-like shape of octagonal symmetry with a diameter of ∼120 nm.

The symmetry of this multi-protein complex (∼110 MDa in vertebrates) results from the fact that it consists of multiple copies of about 30 different proteins called nucleoporins (NUPs). These NUPs are always present in multiples of eight (reviewed in Knockenhauer and Schwartz, 2016; Beck and Hurt, 2017). In total, an NPC of higher vertebrates comprises up to ∼1000 protein molecules, which are organized into complexes depending on their function and localization (Cronshaw et al., 2002; Ori et al., 2014). These complexes and single NUPs can be divided into three main classes: core scaffold NUPs, FG-repeat NUPs and transmembrane (TM) NUPs (also referred to as pore membrane proteins). Overall, the NPC is built of two outer rings, the inner ring, the central channel, the nuclear basket (NB), and the cytoplasmic filaments.

The overall architecture of the NPC and main components are well conserved among eukaryotes, and especially between vertebrate and plant NPCs. On the sequence level, though, the conservation is low (e.g. Tamura et al., 2010; Ori et al., 2013; Obado et al., 2016). Furthermore, most vertebrate NUPs have a homologue in yeast. However, *S. cerevisiae* NPCs show a few differences in NUP composition and are smaller in molecular weight (∼66 MDa) and height due to the reduced stoichiometry of some NUPs (Rout and Blobel, 1993; Yang et al., 1998; Kim et al., 2018; Rajoo et al., 2018).

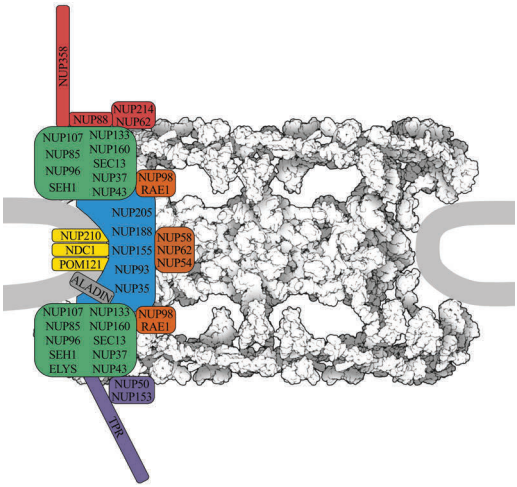

Figure 1.1: The composition of the human NPC embedded in the nuclear envelope. The illustration combines the Cryo-EM maps of the outer ring (PDB 5A9Q) and the inner channel ring (PDB 5IJN). The different groups of NUPs are color-coded; Transmembrane NUPs (yellow), Y complex (blue), FG-repeat NUPs (orange), nuclear basket and associated proteins (purple), cytoplasmic complex (red). Positioning and grouping of NUPs are schematic and not to scale.

The vast majority of NUPs are non-membranous of which most are organized in complexes. They are assigned to the scaffold of the NPC, the permeability barrier in the central channel, the cytoplasmic or the nucleoplasmic parts. In the following section, the main structural blocks of the NPC and the corresponding NUPs are described, with the nomenclature referring to higher vertebrates.

1.3.1 Outer rings and Y complex

The main structural constituent of the outer rings is the Y complex. The Y-shaped arrangement (two arms and a flexible stem) is highly conserved and gives the complex its name (reviewed in Lin et al., 2016). The short arm region of the Y complex is formed by NUP43, NUP85, and SEH1, the long arm region by NUP37 and NUP160, and SEC13, NUP96, NUP107 and NUP133 form the stem region (Van der Walt et al., 2014). The Y complex additionally interacts with ELYS (Lutzmann et al., 2002). ELYS is asymmetrically

localized and therefore only present on the nuclear face. It is connected to the Y complex by directly binding NUP160 (Bilokapic and Schwartz, 2013). Moreover, the cytoplasmic filament component NUP358 acts as a structural element of the outer rings. Two Y complexes stacked on top of each other form one outer ring spoke (Von Appen et al., 2015). Except for ELYS, all components appear iso-stoichiometrically in 32 copies per NPC (Ori et al., 2013).

1.3.2 Inner ring

In addition to the Y complex, the inner ring is the second major constituent of the NPC scaffold. The inner ring complex, also called NUP93 complex, assembles from NUP35, NUP93, NUP155, NUP188, and NUP205 and is the sole component of the inner ring (reviewed in Beck and Hurt, 2017; Lin and Hoelz, 2019). In comparison to the Y complex, whose defined architecture is determined by the interaction of large interfaces, the subcomplexes of the inner ring show a more heterogeneous conformation. This heterogeneity is attributed to the interaction of shorter sequence motifs connected by flexible linkers (Lin et al., 2016).

1.3.3 Central channel and FG-NUPs

The central channel is formed as a consequence of the scaffold NUPs building a ring structure. FG-NUPs line the central channel and harbor interaction sites that anchor them to the NPC scaffold. Their special feature are phenylalanine-glycine (FG) repeat regions that are orientated towards the inner plane. FG repeats are present in different NUPs, most of these are located at the central channel, in particular NUP98 and the NUP62 complex (NUP62, NUP54, NUP58). By assembling, these cohesive FG repeats form the NPC permeability barrier (Ribbeck and Görlich, 2001; Frey and Görlich, 2007). The transport of macromolecules larger than ∼40 kDa by nuclear transport receptors and the free diffusion of small molecules are enabled by interactions of these proteins with FG-repeat domains (Ribbeck and Görlich, 2001; Frey and Görlich, 2007).

1.3.4 Cytoplasmic filaments

The asymmetric NUPs that are exclusively present on the cytoplasmic face of the NPC, are often called cytoplasmic filaments. Their characteristic long and flexible domains point upward from the cytoplasmic Y complex ring into the cytoplasm. The main component of

the cytoplasmic filaments is the metazoan-specific NUP358 (Lin et al., 2016). In addition, the trimeric NUP214 complex, composed of NUP214, NUP62, and NUP88, is also part of the cytoplasmic complex. While not contributing to the filament formation itself, it does harbor domains critical for interactions with other NUPs (Lin and Hoelz, 2019).

1.3.5 Nuclear basket

The NB is a structure of eightfold-rotational symmetry that originates from the outer nuclear ring and converges in a distal basket-like shape (e.g. Cordes et al., 1993, 1997). With its large coiled-coil region, TRP is the architectural element of the NB. Other NB-residing proteins only attach to TPR such as the SENP1, MAD1 and MAD2, GANP, or COP1 (e.g. Yi et al., 2006; Lee et al., 2008; Schweizer et al., 2013). Another binding partner of TPR is the highly mobile NUP153 (Hase and Cordes, 2003; Krull et al., 2004). NUP153 itself is in turn required for the localization of NUP50 (Walther et al., 2001). The only other architectural element of the NB is the protein ZC3HC1, which is needed to keep subpopulation of TPR at the NB Gunkel et al. (2021).

1.3.6 Transmembrane NUPs

In contrast to the high number of non-membranous NUPs, only very few were identified as membrane-spanning proteins. These transmembrane NUPs play a critical role in anchoring the NPC to the NE and influence NE curvature (e.g. Kim et al., 2018). For the vertebrate NPC, three TM NUPs are described; NDC1, NUP210 and POM121. Despite their crucial functions, only NDC1 is universally conserved and is therefore also found in *S. cerevisiae* and fungi (Stavru et al., 2006a; Mansfeld et al., 2006). In contrast to vertebrates, *S. cerevisiae* possesses not three but four TM NUPs (Ndc1p, Pom152p, Pom33p and Pom34p). Considering that TM NUPs are important to anchor the NPC to the NE, it is quite surprising that only NDC1 was found to be universally conserved.

Since the focus of this study is on transmembrane proteins, the individual TM NUPs will, in the following, be discussed in more detail and also put into reference to other species.

NDC1 comprises six N-terminal transmembrane segments with only very short loops. N- and C-terminus are on the cytosol/NPC side, thus making them available for interaction with soluble NUPs (Stavru et al., 2006a). Its largely disordered C-terminal domain is involved

in critical structural interactions with Aladin and NUP155 (Yamazumi et al., 2009; Kind et al., 2009; Mitchell et al., 2010), whereas the C-terminal region contains a binding site for NUP35, important for the substantial recruitment of the NUP93 complex (Eisenhardt et al., 2014). NDC1 is essential in vertebrates and yeast as it is also important for the formation of spindle pole bodies (Winey et al., 1993). Therefore, its loss completely abolishes the assembly of NPCs (Mansfeld et al., 2006; Stavru et al., 2006a; Madrid et al., 2006). Although essential in mammals and yeast, *C. elegans* can survive the deletion of NDC1, though with heavy NPC assembly defects that result in a severe phenotype (Stavru et al., 2006a).

NUP210 is a homodimer-forming type I integral membrane protein with a very large (∼200 kDa) N-terminal immunoglobulin (Ig)-like nuclear domain and a small (∼40 aa long) cytoplasmic C-terminal tail that might be available for interaction with soluble NUPs in vertebrates (Wozniak et al., 1989; Greber et al., 1990; Favreau et al., 2001). NUP210 is generally conserved among most eukaryotes. Even though, the homologues show little sequence similarity, they overall share the same general architecture as they are built from Ig-domains (Antonin et al., 2005; Lin and Hoelz, 2019). The homologue of NUP210 in *S. cerevisiae* is *sc*POM152. Despite its strong conservation, NUP210 is absent in many cell types, e.g., in smooth muscle tissue or fibroblasts (Olsson et al., 2004). The depletion of NUP210 in HeLa cells was found to not disturb NPC formation, thus not essential to this process (Stavru et al., 2006b).

POM121 comprises an N-terminal signal anchor, followed by a large disordered domain and FG repeats, which point into the central channel, and is a vertebrate TM NUP (Hallberg et al., 1993). Mediating the recruitment of other NUPs, POM121 plays a role in anchoring the NPC scaffold by directly binding NUP155 and NUP160 (Antonin et al., 2005; Stavru et al., 2006b; Mitchell et al., 2010). Like NUP210, the lack of POM121 does not negatively affect NPC assembly in postmitotic assembly (Stavru et al., 2006b). Interestingly, even after co-depleting these two TM NUPs, formation of functional NPCs is still possible (Stavru et al., 2006b).

1.4 NE and NPC breakdown and reassembly

In organisms that undergo open mitosis, cells disassemble their nuclei during mitosis, and NE and the NPC reassemble upon return to interphase. During nuclear envelope breakdown

(NEBD), membranes are detached from chromatin and membrane proteins of the NE get dispersed into the connected ER. Moreover, NUPs become soluble as a result of the disassembly of the NPCs. During NEBD, the disruption of protein-protein interactions within NPCs and to the nuclear lamina, as well as between NE proteins and chromatin, ensures access for the chromosome segregation machinery (Linder et al., 2017). These processes are orchestrated, among others, by the phosphorylation of NUPs, which blocks their interaction sites.

Therefore, the reassembly process involves the recruitment of membrane vesicles and membrane proteins embedded in the ER to chromatin, membrane fusion, and incorporation of NPCs from soluble NUP complexes (reviewed in Kutay and Hetzer, 2008). As mitosis completes and NE remodeling is initiated, dephosphorylation is one of the critical first steps taking place to enable re-recruitment of NE and NPC components to the chromatin. Proteins are re-recruited in a coordinated process to avoid premature NE closure. As the NE membrane reemerges from the ER, NETs are also recruited again, contributing to the reassembly process (Antonin et al., 2008).

1.4.1 NEBD and NPC disassembly upon mitosis entry

The disassembly of NPCs constitutes an initial step in NEBD and is mediated by intensive phosphorylation of NUPs. Hyperphosphorylation of NUP98 at more than 15 sites by multiple kinases, including cyclin-dependent kinase 1 (CDK1), polo-like kinase 1 (PLK1), and NIMA-related kinases (NEKs), marks the initial event in NPC dismantling during prophase (Laurell et al., 2011). This leads to the disintegration of the permeability barrier. As an inner ring component, the phosphorylation of NUP35 at its disordered regions interrupts the interaction with NDC1. This causes the disintegration of membrane contacts and destabilizes the NPC scaffold, which, in turn, results in the dismantling of the inner ring components. As a consequence, the association of NUP35 with NUP93 and NUP205, as well as the anchoring of the NUP62 complex, is already impaired in the early prophase and serves as a trigger for further NPC disassembly (Eisenhardt et al., 2014; Linder et al., 2017). The dismantled complexes do not all disperse into single NUPs, but rather into smaller subcomplexes that might facilitate reassembly after mitosis. After NPC disassembly, the overall NE structure is subsequently reorganized, due to a strong increase in kinase activities (Gerace and Blobel, 1980). This leads to a phosphorylation-driven depolymerization of the nuclear lamina and inhibition of the phosphorylation-dependent binding of chromatin-binding proteins, such as

BAF, which ultimately results in the dissociation of chromatin from the NE (Beaudouin et al., 2002; Gorjánácz et al., 2007). Various other INM proteins are released from lamin and chromatin, such as LBR, and redistribute into the interconnected ER (Courvalin et al., 1992; Champion et al., 2017). Spatial segregation of chromatin and membranes is further promoted during prometaphase by pulling forces from the microtubules and the LINC complex. While most of the NPCs are disassembled in metaphase, NUP210 seems to withdraw quite late and was shown to still be associated with NE in late metaphase (Galy et al., 2008). To prevent ectopic NPC assembly and premature association of NUPs with chromatin, NUPs remain phosphorylated during mitosis. Additionally, NUPs, as well as, for example, LBR and lamin B are bound by the nuclear transport receptor importin β, which prevents proteins from interacting with each other (Harel et al., 2003).

1.4.2 Postmitotic NE formation and NPC reassembly

After chromosome segregation, an essential step is to spatially separate nucleo- and cytoplasmic components from each other to rapidly establish a functional compartmentalization. Until anaphase, the mitotic ER is kept away from chromosomes and spindle area, but upon mitotic exit, nuclear reformation takes place at two distinguishable sites (reviewed in Otsuka and Ellenberg, 2018). The regions in which the sister chromatids are pulled to the opposite poles are initially kept membrane-free. At these so-called core regions, NPC assembly is delayed. In the non-core regions though, more towards the periphery, ER membranes start to re-emerge, re-enclosing chromatin and NPC assembly is initiated. As a consequence, NPC formation after mitosis differs from interphase NPC assembly not only in localization but also in the underlying mechanism (reviewed in Kutay et al., 2021; Otsuka et al., 2023).

In postmitotic assembly, membrane contacts to chromatin are established by INM proteins such as LBR, emerin, and Sun2, which can directly bind to chromatin (e.g. Ellenberg et al., 1997; Haraguchi et al., 2000; Anderson et al., 2009). Subsequently, chromatin is covered by highly fenestrated ER sheets that can expand laterally from the initial contact points (lateral expansion model), or cover chromatin in several layers of ER sheet (cisternal stack model) (reviewed in Wandke and Kutay, 2013; Schellhaus et al., 2016; Otsuka and Ellenberg, 2018). The discontinuities in the ER shrink as NE formation proceeds. According to the current state of scientific knowledge, these discontinuities are presumably functioning as a seeding point for NPC formation, initiated by the recruitment of ELYS to these venues (Walther

et al., 2003a; Franz et al., 2007; Rasala et al., 2008).

Postmitotic NE/NPC formation is triggered by a drop in CDK1 activity with a concomitant increase in protein phosphatase activity of PP1 and PP2A, reverting the phosphorylation events that took place upon mitosis entry (Huguet et al., 2019). Reformation of the NE/NPCs proceeds with increasing accessibility of chromatin and further release of NUPs from importin β.

Interestingly, the information on where the chromatin is located is conveyed by the small GTPase Ran. The guanine nucleotide exchange factor for Ran, RCC1, is associated with chromatin and therefore generates RanGTP in the vicinity of chromatin. RanGTP, in turn, binds to importin β, displacing NUPs and thereby abolishing the chaperoning effect on NUPs close to chromatin, making them available for interactions (Walther et al., 2003b; Forbes et al., 2015).

While it is still a matter of debate which structural intermediates of the NPC exist, the order in which NUPs are recruited has been quite well established. With the initial binding of ELYS to chromatin in late anaphase, the Y complex components are also entrained, leading to the reassembly of the NPC scaffold. The Y complex recruits the TM NUP Pom121 and NUP35, the latter of which, in turn, attracts TM NUP NDC1 (Antonin et al., 2005; Mansfeld et al., 2006; Mitchell et al., 2010; Rasala et al., 2008; Vollmer et al., 2012). Subsequently, NUP155 is recruited by NUP35 (Eisenhardt et al., 2014). NUP35 is further required for recruitment of the inner ring components NUP93, NUP205 and later the central channel NUP62 complex, as well as NUP98 (Dultz et al., 2008; Vollmer et al., 2012; Vollmer and Antonin, 2014). Part of the peripheral NPC components, NUP153, appears to also re-associate during late anaphase, whereas NUP214, NUP358, and TPR are incorporated later in telophase probably concomitantly with NUP210 (Antonin et al., 2005; Vollmer et al., 2015; Otsuka et al., 2023).

After NPC reassembly, NE sealing is mediated and controlled by ESCRT-III (Olmos et al., 2015; Vietri et al., 2015). NE/NPC reformation and the establishment of transport competence are completed 10 minutes after onset of anaphase (Dultz et al., 2008).

1.4.3 Interphasic NPC *de novo* assembly into the NE

Interphase NPC assembly exhibits several major differences compared to postmitotic NPC assembly. Since interphase NPC assembly starts in late telophase, the incorporation of NPCs