Chapter 1

The Nuclear Pore Complex structure and the transport through it

An Introduction
**NPC Structure**

In eukaryotic cells, a double lipid bilayer known as nuclear envelope (NE) surrounds the cell’s nucleus. The NE is equipped with Nuclear Pore Complexes (NPCs) to allow passage of macromolecules and to regulate accessing the DNA. This structure of the cells (50 MDa in yeast and 120 MDa in vertebrate) has a cylindrical shape with octagonal symmetry. It functions to regulate the traffic across the NE. The NPCs are composed of several proteins called nucleoporins (Nups) that are present in multiple copies. Using biochemical, biophysical, computational modeling, and super-resolution microscopy techniques, past research has revealed for many Nups their structure and position in a subcomplexes, and several models have been proposed to describe how the complexes are assembled to form the NPC (reviewed in Hurt and Beck, 2015).

The overall structure of the NPC is conserved between yeast and vertebrate cells. The yeast NPC is ±100 nm in diameter and ±40 nm in height, and the corresponding sizes in vertebrate are ±130 nm and 80 nm, respectively (Yang et al., 1998). The hole in the centre of the pore of yeast has a diameter of ±40 nm. Structurally, the yeast NPC can be categorized into 6 subcomplexes, pore membrane (POM) proteins, cytoplasmic filaments, scaffold Nups for the outer and inner rings, linker/adaptor Nups, the FG-repeat Nups and the nuclear basket (see Figure 1.1) (Alber et al., 2007b, reviewed in Grossman et al., 2012). The pore membrane subcomplex, also known as the Ndc1-complex is built by three membrane proteins, Ndc1 (nuclear division cycle 1), Pom (Pore Membrane protein) 34 and Pom152. Pom33 (not shown in the Figure 1.1) is a transmembrane protein that dynamically associates with NPCs and is involved in NPC distribution as assembly (Chadrin et al., 2010). POMs connect the NPC to the NE by interaction with the core components of the NPC (Alber et al., 2007a,b). Ndc1, the only conserved pore membrane Nup, contains 6-transmembrane domain at its N-terminus and an α-helical domain at the C-terminus. To form the complex, the cytosolic domain, located at the very beginning of Ndc1, interacts with the first 170 residues of Pom152 that are exposed to the cytoplasm; the latter also interacts with the soluble domain at the N-terminal of Pom34 (Onischenko et al., 2009). To anchor the NPC in the NE, Ndc1 interacts with the core scaffold of NPC via Nup53 or Nup59 (Nup35 in vertebrate) (Onischenko et al., 2009).
Figure 1.1. The structure of Nuclear Pore Complex and the folding of each of the nucleoporins. The nucleoporins and their homologs (in vertebrate and plants) are classified as subcomplexes. The protein folding is indicated on the left side of the Nups. This figure is taken from Grossman et al., 2012 and duplicated with permission from the authors.

The inner rings

The core scaffold of NPC forms two inner rings that are sandwiched by two outer rings. The inner ring of the NPC is built by the larger Nups, namely Nup192, Nup188, Nup170 and Nup157, and the linker Nups, Nic96 and Nup82, that act as adaptors to connect the scaffold Nups to the FG-repeat Nups (Alber et al., 2007b; Jeudy and Schwartz, 2007; Schrader et al., 2008). Two inner rings subcomplexes are located closely to each other at the axial plane of the NPC and are connected to the membrane Nups directly.

Recent work revealed that the largest Nups, Nup192 and Nup188, form a twisted S-like shape structure with a \( \alpha \)-selenoid fold. The C-terminal domain of Nup192 has a rod-like structure (Amlacher et al., 2011; Schrader et al., 2008; Flemming et al., 2012; Andersen et al., 2013; Sampathkumar et al., 2013; Stuwe et al., 2014). Interestingly, the fold structure of Nup192 and Nup188 is similar to that of nuclear transport factors (NTRs) and suggests that the NPC and NTRs family are evolutionary related (Flemming
et al., 2012; Andersen et al., 2013; Stuwe et al., 2014). Nup192 is essential in yeast and Nup188 is not, but both are important to maintain the permeability of the pore. Knock down of Nup192 in *Saccharomyces cerevisiae* causes an increase in pore permeability similar what is observed for the strains Nup188Δ and Nup170Δ (Shulga et al., 2000; Theerthagiri et al., 2010; Sampathkumar et al., 2013, Stuwe et al., 2014).

Nup157 and Nup170 consist of β-propeller domains at the N-terminus and two different packaging α-selenoids at the C-terminus, resulting in an overall structure like a C-letter with an additional extended long α-superhelical stack (Flemming et al., 2009; Seo et al., 2013; Whittle and Schwartz., 2009). The different domain structures of the inner ring Nups are thought to provide large flexibility to the structure so that the structure can dilate and constrict to adjust the size of the central channel. This may allow passage of large cargoes such as ribosomal subunits (Sampathkumar et al., 2013).

Nup170 also plays a role in NPC assembly. The C-terminus functions as the anchor point to the NE, whereas the N-terminus is responsible for recruiting Nup188, Nup159 and Pom34 (Flemming et al., 2009). Depletion of Nup170 and Nup157 in yeast causes a lower NPC number, defects in nuclear transport and mislocalization of other Nups (Makio et al., 2009). Nup157 and Nup170 are involved in chromatin reorganization and gene expression in yeast, although the mechanisms are still unclear (Van de vosse et al., 2013). The finding that the C-shape domain of Nup157 is able to bind the dsDNA and ssRNA in a sequence-independent manner strengthen the idea of its involvement in regulation of gene expression (Seo et al., 2013).

*The outer rings*

The outer ring is formed by a heptameric subcomplex, known as the Nup84 complex (a nanomeric complex in vertebrate, Nup107-160 complex), in baker’s yeast consisting of Nup120, Nup85, Seh1, Sec13, Nup145c, Nup84 and Nup133 (Siniossoglou et al., 1996, 2000; Lutzmann et al., 2002) (Figure 1.2). This complex is a very stable and can be expressed, purified and reconstituted in vitro (Siniossoglou et al., 2000; Lutzmann et al., 2002). Electron microscopy reveals that the Nup84 complex has a Y-shape with the long and short arm, respectively, formed by Nup120 and Nup85-Seh1, while Nup145c, Sec13, Nup84 and Nup133 form the main stalk (Siniossoglou et al., 2000; Lutzmann et al., 2002). The structure of this complex has been resolved at atomic level. The components of the Nup84 complex have β-propellers and α-selenoid
folds (Hsia et al., 2007; Brohawn et al., 2009; Nagy et al., 2009; Berke et al., 2004; Boehmer et al., 2008; Debler et al., 2008; Seo et al., 2009, Leksa et al., 2009; Liu et al., 2012). The arrangement of beta propeller and α-selenoid folds is similar to that found in COPII membrane vesicles and it has been hypothesized that NPC and COPII membrane vesicle share a common ancestor (Devos et al., 2004). hNup133 has been shown to act as a membrane curvature sensing ArfGAP1 lipid packing sensor (ALPS) and ALPS motifs have also been predicted the yeast Nup120 and Nup133, located at one arm and at the end of the main stalk of the Y-complex, respectively (Kim et al., 2014; Shi et al., 2014; Drin et al., 2007; Drin and Antonny, 2010).

Recent papers by Stuwe et al., 2015 and Kelley et al., 2015 showed how the hub of the Y-shaped complex is formed. It is built by interaction between the C-terminal domain of Nup120, with Nup85 and with Nup145c. How the Nup84 complex is arranged in the pore is still under debate (Figure 1.2). Firstly, the Rout lab postulated a computational model based on an analysis of the spatial constraints derived from immunoEM data, measurements of the approximate shape of the Nups, the stoichiometry of the Nups and pull-out experiments identifying neighboring proteins. This model features sixteen copies of the Nup84 complex per NPC. The sixteen Nup84 complexes form two rings located at the nucleoplasmic and cytoplasmic sides of the pore (Alber et al., a, b 2007). A second model, the Fence-like model was proposed based on the basis of the hetero-octameric crystal structure of Nup85-Seh1 and Nup145c-Sec13. The Fence-like model states that the outer rings are formed by 32 copies of the Nup84 complex with head to tail arrangement (Hsia et al., 2007; Debler et al., 2008), as also proposed for the human Nup107-160 complex (Bui et al., 2013). The third (lattice) model is based on the homology of the Ancestral Coatomer Element 1 (ACE1) protein in the NPC and proteins of COPII vesicles. The assembly of Sec13-Sec31 as the outer component of COPII vesicles resembles how Sec13 interacts with Nup145c and Seh1 with Nup85. Both Nup145c and Nup85 together with Nup84 and Nic96 are defined as ACEI proteins and have a similar α-helical structure as Sec31 (Brohawn et al., 2008). A head to tail arrangement fits well with recent findings on cryo-Electron Tomography and super-resolution microscopy images in the human NPC ( Seo et al, 2008; Szymborska, et al., 2013; Bui et al., 2013; Maimon et al., 2012).
The FG-Nups

At the central channel of the NPC, FG-repeat (Phenylalanine-Glycine repeat) rich proteins form the selective barrier. Different types of FG Nups motif are present based on typical sequences such as the FxFG motif in Nsp1 and GLFG in Nup100. For the major part of their sequence the FG-Nups lack a stable secondary or tertiary structure. Parts of the FG-Nups form small structured domains known as β-sandwich or RNA recognition motif (RRM) followed by a coiled-coil or α-helical fold structure (Melcak et al., 2007; Devos et al., 2006) to anchor the proteins in the scaffold of the NPC. In total, 12 FG-Nups were identified in yeast (11 in vertebrate) (Alber et al., 2007b) and they can be classified as three types of FG-Nups based on their location in the centre or at the cytoplasmic and nucleoplasmic sides of the pore.

Two types of Nsp1 subcomplexes exist, one at the centre (Nsp1, Nup49 and Nup57) and one at the cytoplasmic side (Nsp1, Nup159, Nup116 and Nup42). Both are connected to two copies of Nic96 with additional interactions to Nup82 for the one that is facing the cytoplasmic side (Alber et al., 2007b). Contrary, Nup145N, Nup60 and Nup1 are connected directly to the inner ring components similar to that of Nup53 and...
Nup59, which are facing toward the pore membrane. Nup2, a homolog of Nup1, also contains FG-repeat motifs and is located mainly on the nucleoplasmic side of the NPC. It is classified as a shuttling Nup, because it goes on and off from the nuclear basket and travels between the cytoplasmic and nucleoplasmic side in a Ran-dependent manner (Dilworth et al., 2001). The FG-repeat Nups function as the permeability barrier by interacting to the nuclear transport factors (NTFs) with low affinity. Typically, deletion of only single non-essential FG-repeat Nups is not lethal, possibly because other FG-Nups can replace them but typically reduces the overall transport of soluble and membrane proteins (Meinema et al., 2011; Popken et al., 2015)

Nucleocytoplasmic Transport

Soluble macromolecules can pass the pore via two mechanisms: passive diffusion and active transport; beside metabolites and ions, small molecules up to 40-60 kDa are freely diffusing across the NPC in few minutes time scale. Nevertheless, some has reported that yeast’s NPC could accommodate passage of cargo larger than 60kDa in longer period of time (Shulga et al., 2000; Popken et al, 2015) as well as in higher eukaryotic cells (Chatterjee et al., 1997; Lénár and Ellenberg, 2006) For entry of much bigger cargoes and to accumulate nuclear-destined proteins, active transport comes to play, which is mediated by transport factors (importins) of the karyopherin family. This active transport depends on a Ran gradient, where Ran in the GTP-bound form is present abundantly in the nucleus (maintained by Ran exchange factor (RanGEF), Rcc1 in vertebrate), while the GDP-bound form is in the cytoplasm (maintained by Ran GTPase-activating protein (RanGAP)).

Protein of the Karyopherins β family (Kapβs) translocate the cargoes by interacting to the FG-repeat Nups at the central channel. In a few cases, Kapβ (classified as Kapβ1, Kap95 in yeast) cannot recognize the cargo directly and adaptor proteins are needed. One of them is Kapα (Kap60 in yeast, and six isoforms of Kapα in vertebrate). Kapα recognizes the cargo by interacting to the nuclear localization signal (NLS, see below for detailed information). The NLS-containing cargo-Kapα-Kapβ1 trimeric complex travels through the pore (see below for detailed information). Once they reach the nucleoplasm, Ran-GTP binds to Kapβ, which causes a conformational change that releases cargo and Kapα. This process is known as the classical import pathway (Figure
Kapα is exported out from the nucleus by RanGTP-bound Cse1 (Cse1: exportin of Kapα) (Matsuura and Stewart, 2004). At the cytoplasm, the complexes disassembles by hydrolysis of the Ran-bound GTP releasing Kapα from the exportins (Figure 1.3).

Figure 1.3 Nucleocytoplasmic transport scheme. The import and export of the cargo molecules is driven by the concentration gradient of RanGTP that is maintained over the nuclear envelope by RanGEF and RanGAP. Active import of nuclear-destined cargoes is facilitated by importins, which in some cases requires an adaptor to recognize the cargo. The dimeric or trimeric complexes formed in the cytoplasm travels through the NPC. At the nucleoplasmic side, the complex is dissociated by binding of RanGTP. Nuclear export is orchestrated in a similar way but here an exportin recognizes the nuclear export signal (NES) and the exportin-Cargo-RanGTP complex crosses the NPC. The complex dissociates in the cytoplasm after hydrolysis of Ran-bound GTP. This figure is taken from Grossman et al., 2012 and duplicated with permission from the authors.
The “zip-code” or signal sequences for nuclear-destined protein are diverse as are the importins. For the classical import pathway, the NLSs are defined as stretch(es) of positive amino acids (arginine and lysine) that are present in the body of protein. The first NLS was found in 1982 from Simian Virus 40 (SV40) large T-Antigen. It contains one cluster of basic region (PKKKRV) later on known as the classical NLS (Colledge et al., 1986). Later on Dingwall et al., 1988 found the NLS of nucleoplasmin from Xenopus, which contains two stretches of positive residues spaced by flexible residues (KRPAATKKAGQAKKK). Deletion of one of the basic residues resulted in targeting failure; this sequence was called a bipartite NLS.

More than three decades after the discovery of the first NLS, large numbers of NLSs, especially in yeast (Hahn et al., 2008) are known (reviewed in Marfori et al., 2011). For example, initially the bipartite NLS was defined as two clusters of basic residues separated by a spacer of 10-12 residues, but other studies revealed that the separation could be even larger than 40 amino acids (Lange et al., 2010; Giesecke and Stewart., 2010).

When the crystal structure of Kap60 and Kap60-NLS complexes was deciphered, it enlightened the mechanism for NLS-containing cargo recognition (Conti et al., 1998, Conti and Kuriyan, 2000). Kap60 contains 10 tandem Armadillo repeats organized in right-handed superhelix that form a banana-like structure (Conti et al., 1998) with two shallow binding grooves. The minor (res. 331-417aa) and major (res. 121-247aa) grooves are formed from an array of tryptophan and asparagine residues. The NLS binds antiparallel to the Kap60 direction. Each binding site has specific/favorable residue for binding the NLS, namely arginine at P2′ position (minor binding site) and lysine at P2 position (major binding site) (Marfori et al., 2011).

At the N-terminus of Kapα, a 40-residue long unstructured domain contains conserved basic amino acid called importinβ-binding domain (IBB domain), which acts as an auto-inhibitory domain of Kapα and competes with NLSs for docking into the binding site (Görlich et al., 1996; Cingolani et al., 1999, Kobe, 1999). When Kapα is alone, the IBB domain is bound to the NLS binding site. The binding affinity of IBB for the binding site on Kapα is relatively low (Kd = 4 µM) compared to the affinity of the IBB domain for the binding site on Kap95 (Kd = 11 nM). Therefore, in the presence of an NLS-cargo a conformation is favored where the IBB domain is displace from the Kap60 binding site by the NLS, and the IBB domain binds Kap95 (Catimel et al., 2001). In the
nucleus, Nup2 is helping the Kap60 to release the cargo by competing for the minor binding site of the Kap60, therefore Nup2 also accelerates the cargo-releasing process (Matsuura et al., 2003; Gilchrist et al., 2002).

The Kapβ1 is built by HEAT-repeat motifs organized into a left-handed superhelix composed of IBB-, Ran- and NPC-binding domain (Cingolani et al., 1999). At the inner surface of Kapβ1 there are an IBB-domain binding site at the C-terminus and a Ran-binding domain at the N-terminus, while the FG-repeat Nups interaction regions are on the surface of the protein (Kutay et al., 1997; Chi et al., 1997). The third phenylalanine residue in the FxFG and GxFG motifs interacts with the hydrophobic region on the surface of Kapβ1. The binding is of low affinity, allowing Kapβ1 to bind and release multiple FG-repeat Nups until it reaches the nucleoplasm. The IBB-domain changes its conformation from unstructured to helical structure when bound to the Kapβ1 (Cingolani et al., 1999).

The mechanism how proteins traffic across the NPC is unclear. Several models have been postulated:

First, the Brownian-affinity gating or virtual gating was postulated by Rout et al., 2000. In this model the initial entry of cargo depends on the Brownian motion and the filamentous FG-Nups form an entropic barrier for large molecules whereas small ones can freely pass. The entropic barrier is compensated by the enthalpy component, which results from binding of FG-Nups and transport factors. This allows transport of large molecules aided by transport factors.

Second, the selective phase or hydrogel model by Ribbeck and Gorlich, 2002 postulates that a diffusion barrier is formed by cohesive hydrophobic interactions of numerous copies of FG-Nups, which results in a hydrogel-like structure (Frey et al., 2006). The pores within the hydrogel are small enough to exclude cargo larger than 40kDa, much like a molecular sieve. In contrast, cargo-bound to Kap (Mw > 90kDa) are able to diffuse through the hydrogel by melting the FG-Nup interactions (Ribbeck and Gorlich, 2002; Frey and Görlich, 2007).

Third, the reduction of dimensionality (ROD) model postulated by Reiner Peters proposes that FG-Nups coat the wall of the NPC and form a coherent hydrophobic layer. This model is based on the finding that NPC are saturated by transport factors that bind to the FG-Nups. The transport factors bound FG-Nups cause the FG-Nups filaments to collapse towards the side of central channel and reduce their dimension. As a
consequence, a hole is formed in the centre of the NPC where molecules can pass through. Thus, cargo-bound transport factors reach their destination via a two-dimensional random walk by binding and unbinding to the FG-Nups (Peters, 2005; Peters, 2009).

Fourth, Rexach and colleagues proposed the Forest model (Yamada et al., 2010), a combination of the virtual gate and selective phase model. This model is based on the finding that FG Nups are present as globular collapsed coils and extended collapsed coils, together forming a forest-like landscape and creating two distinct transport zones. The collapsed coiled domains of FG-Nups are located away from the anchor points and connected by extended coiled domains (“the trunk of the tree”), which results in a small hole in the middle of the pore that allows passage of molecules in a fashion similar to the hydrogel model (zone 1). The globular collapsed coil FG-Nups (called “shrub”) are located adjacent to their anchor point in the wall of NPC together with the “trunk of the tree”, creating a virtual gate for small macromolecules (zone 2) (Yamada et al., 2010).

Future models will have to include that the presence of transport factors greatly impacts the structure of the FG-Nups, such as highlighted in (Lowe et al., 2015) and the Dirty Velcro model by Lim and colleagues (Schleicher et al., 2014).

**Transport of Inner Nuclear Membrane Proteins**

Membrane proteins that function at the inner membrane cross the nuclear envelope through the NPC. Their sorting starts already at the point of synthesis and membrane insertion. Membrane insertion is mediated by the Sec61 translocon and the Guided-Entry of tail-anchored (GET, initially stand for Golgi ER trafficking) pathways (review in Shao and Hedge, 2011; Denic, 2012). Beside the GET pathway and the Sec61 translocon, other insertion machineries exist such as Sec62-Sec63 and Hsc70/Hsp40 (Jung et al., 2014), but little is known about their specificity and functioning. The Sec61 translocon inserts monotopic transmembrane domain (TMD) with long luminal domains and polytopic TMDs post- and co-translationally, respectively. When these proteins are still synthesized in the ribosome, the signal recognition particle (SRP) recognizes the hydrophobic residues of a signal peptide at the N-terminus of the nascent chain. The Alu-domain of the SRP located near the elongation site of the ribosome slows down the translation. Thus, the SRP bring them close to the ER where
the SRP receptor and Sec61 translocon reside. Next, the hydrophobic domain is transferred to the Sec61 system and inserted into the ER membrane. Monotopic membrane proteins with short luminal domains (tail-anchored membrane proteins) or proteins with the TMD located at the very C-terminus are inserted via the GET pathway post-translationally (Schuldiner et al., 2008, review in Borgese and Fasana, 2011). In yeast, the Get complex consists of Get1, Get2, Get3, Get4 and Get5 (Schuldiner et al., 2005; Jonikas et al., 2009). Get3 (Asna1/Trc40 in mammalian) is a soluble ATPase and acts as the chaperone shielding the TMD at the end of translation, and Get1/Get2 acts as the receptor of Get3, which resides in the ER. The Get4/Get5 complex makes physical contacts with Get3 and has weak interactions with the ribosome (Fleischer et al., 2006). It is believed that the Get4/Get5 complex functions to bring the tail-anchor protein to Get3.

Figure 1. 4 Proposed mechanisms how inner nuclear membrane proteins travel to the inner nuclear envelope. This figure is taken from Katta et al., 2014 and duplicated with permission from the authors.
The inner nuclear membrane proteins (INMp) play important roles in maintaining nuclear integrity, chromatin remodeling, NPC assembly, DNA repair and transcription control (Review in Starr and Fridolfson, 2010; Mikhail and Moazed, 2010; Rothballer and Kutay, 2013; Meister and Taddei, 2013). Some of the INMps interact with nuclear lamina, a meshwork of proteins located adjacent to the INM, which is present in eukaryotic cells, but not in baker’s yeast. Mutations in the lamina or lamina-associated proteins are linked to tissue-specific diseases such as muscle diseases, partial lipodystrophy syndromes, peripheral neuropathy, laminopathies and progeria (Schreiber and Kennedy, 2013; Mendez-lopez and Worman, 2012; Worman and Courvalin, 2005). LEM (Lap2β-Emerin-Man1) family proteins are found to interact with the chromatin via barrier-to-autointegration factor (BAF, an essential protein in metazoa) (Wagner and Krohne, 2007). Lamin also functions in gene silencing by tethering the chromatin close to the INM. SUN (Sad1-Unc84-homolog)-family proteins are responsible for connecting the nucleoskeleton to the cytoskeleton via the KASH domain of nesprin, forming a so-called linker nucleoskeleton and cytoskeleton (LINC) complex that functions in nuclear migration, anchorage and chromatin positioning (Rothballer and Kutay, 2013).

The importance of INM components in cellular life has triggered scientists to investigate how transport occurs. The first mechanism was postulated by Soullam and Worman, 1993 when they observed the Lamin Beta receptor (LBR) protein to travel from the ER, where the protein is inserted after translation, to the nuclear envelope. LBR has a multispansning TMD and an N-terminal ~230 residues (~20 kDa) extralumenal domain and LBR contains a targeting signal that is located at the N-terminus in the first TMD (Soullam and Worman, 1993; Smith and Blobel, 1993). Using photobleaching techniques to investigate the mobility of LBR at the NE, the authors showed that the LBR is immobile, whilst it is highly mobile at the ER, suggesting that LBR has interaction partners at the INM likely chromatin or lamin (Ellenberg et al., 1997). LBR failed to target to the NE when the extralumenal domain was increased to 70 KDa, suggesting there is maximum limit for size of the extralumenal domain of membrane proteins destined for the INM (Soullam and Worman, 1993; Soullam and Worman, 1995). The ultrastructure of the NPC shows lateral channels that can accommodate extralumenal domains of sizes up to ~10nm, which are in accordance with these data (Bui et al., 2013; Maimon et al., 2012). Thus, the diffusion retention
model states that INMp can freely diffuse from ER to ONM and use the lateral channel to reach the INM, where they then stay by interacting to nuclear components (Figure 1. 4). Recently convincing evidence was presented that the human proteins, LBR, emerin and Sun2 are using the diffusion-retention pathway (Boni et al., 2015; Ungricht et al., 2015).

The second mechanism, which is studied in this thesis is a mechanism that depends on energy input from the gradient of RanGTP. It was first proposed based on the observation of the energy-requirement and temperature-dependence of the transport of Lap2β (Ohba et al., 2004). Later on, King et al., 2006 showed that the localization of Src1/Heh1 (helix-extension-helix 1) and Heh2 (helix-extension-helix 2), two INMp in yeast, depend on the presence of specific Nups, Kap60 and Kap95, and a functional Ran gradient. They proposed a mechanism similar to that of soluble proteins. A metazoan INM protein with characteristics similar to that of Heh1 and Heh2 is the INM-localized protein POM121 (Funakoshi et al., 2011; Yavuz et al., 2010; Kralt et al., 2015). As the Kap-cargo complexes are much larger than the proposed size limit of the lateral channels, a transport route distinct from passive diffusion seemed most likely (Figure 1. 4).

Different from yeast cells that have a closed mitosis, the NE of metazoan cells ruptures during cell division in a process called open mitosis. Therefore, in metazoan cells there are two possible ways for transporting the INMp. First, during interphase, where the NE is intact, the membrane proteins must pass the NPC to reach the INM. Second, the membrane proteins may be recruited to the chromatin when the NE reassembles and hence arrive at the INM in an NPC transport-independent fashion. The studies in yeast described in this thesis may thus have relevance for the targeting of membrane protein in interphase cells or in post-mitotic human cells.

The last mechanism proposed for targeting of INMps is an NPC-independent pathway that relies on vesicle trafficking. Herpes viruses usually utilize a vesicle-based mechanism to invade cells (Kobiler et al., 2012). From the ER, the viruses are encapsulated into membrane vesicles that bud off from the ONM, and pass via the luminal space after which they are integrated in the INM. The mechanism is not limited to viral systems as the nuclear export of a messenger ribonucleoprotein (mRNA) also occurs independent from the NPC (Mattenleitter et al., 2013; Speese et al., 2012).
Research Aims and Outline of the thesis

We aimed to reveal the mechanism of transport of the Heh1 and Heh2 proteins to the inner nuclear membrane in baker's yeast.

1. King et al, 2006 showed that transport of Heh1(Src1) and Heh2 is dependent on Kap60/95, RanGTP, Nup170 and Nup2, and both proteins contain NLS sequences. However, an NLS sequence is not sufficient to target a membrane protein to the INM and hence we asked what other factor(s) are used for the proper sorting of a membrane protein to the yeast INM? In chapter 2, we created a reporter system to address which sorting signals are required for accumulation of membrane proteins at the INM. My specific contributions were in studying the h2NLS characteristic, production and purification the h2NLS-L region for chromatography analysis, Stokes radius measurement, H-NMR and 1H-13C HSQC-NMR. Furthermore, I also demonstrated the very high affinity of h2NLS to its transport factor Kap60 compared to a classical NLS and showed that the Heh1 NLS-L sequence can replace the corresponding domain within Heh2 for INM targeting.

2. A large number of NLSs of soluble proteins have been characterized (review in Marfori et al, 2011) but none are from integral membrane proteins. As we found a very high affinity between the Heh2 NLS (later on termed h2NLS) and Kap60, we wondered whether the NLSs of membrane and soluble proteins differ. In Chapter 3, in a collaboration with the Cingolani lab, we address how and why the h2NLS and h1NLS bind to Kap60 with such a high affinity. Complementing the in vitro studies by the Cingolani lab, I demonstrated that mutation of a single amino acid in the h2NLS had dramatic effects on the subcellular localization of soluble or transmembrane proteins, and I showed that the mutant NLSs were compromised in recruiting Kap60 in vivo. A comparison with the NP-NLS confirmed that the Heh2 NLS has distinct characteristic compare the classical bipartite NLS.

3. In Chapter 4, I investigate the dependence of active membrane protein import to the INM on the scaffold Nups, particularly the Nup84-complex, using a set of strains lacking domains of components of the Nup84 complex (Fernandez-Martinez et al, 2012). Using a Heh2-derived reporter, I showed that almost all Nup84 complex components are important for the import of the membrane
proteins but less so for transport of soluble proteins carrying the same NLS. In those strains where the connectivity of the NPC scaffold is most affected by the domain deletions, the impact on INM import is strongest. The accumulation of the Heh2-derived reporter proteins at the NE also correlates with the fitness of the cells, a correlation that was not found for the soluble reporter proteins.

4. **In chapter 5** I show that cells overexpressing the Heh2-derived reporter and GFP-Heh2 have deformed nuclei and show a growth defect. Both phenotypes are specific for overexpression of NLS-L containing membrane proteins, and are not observed when overexpressing proteins lacking a transmembrane domain, an NLS or ID linker. Different hypotheses have been tested to try to explain these observations but a final answer awaits further experiments.

5. In chapter 6, we discuss the experiments and conclusions described in this thesis and give a perspective of future research on the topic of transport of membrane proteins to the INM.
References


