1. Introduction

One of the most fundamental principles of life is the ability to store, process and transmit the information required to synthesize the macromolecular building blocks, perform the biochemical reactions and coordinate the cellular processes that constitute the cell. Although all cells store this information in form of DNA, transcribe it into various forms of RNA (in particular rRNAs, mRNAs, tRNAs or regulatory RNAs) and eventually translate some of it into proteins (with mainly architectural or enzymatic functions), the spatiotemporal arrangement of this flow of information shows remarkable differences in pro- and eukaryotes. This is due to the fact that eukaryotes contain, amongst many other sub-cellular compartments, a cell nucleus that hosts the genome.

The nucleus is delimited by the nuclear envelope (NE), a double membrane that is continuous with the endoplasmic reticulum (ER). Consequently, the key processes of transcription (in the nucleus) and translation (by cytoplasmic ribosomes) are separated by a physical barrier in eukaryotic cells, no longer allowing the immediate co-transcriptional translation of an emerging transcript into protein. Rather, an additional exchange step between nucleus and cytoplasm is required to circumvent an interruption of the continuous flow of information in eukaryotes. Amongst the even more profound consequences of arranging the genetic information into a nucleus is the need for a fundamentally new mechanism to segregate the duplicated DNA after replication (i.e. mitosis), as the DNA is no longer attached to the cytoplasmic membrane and thus cannot simply be segregated by surface membrane motors anymore (Cavalier-Smith, 2010b).

Yet, the cellular sub-organization into a nuclear and cytoplasmic compartment clearly offered manifold opportunities in regard to genome maintenance and regulation of gene expression. Firstly, the confinement of the genetic information into an enveloped and mechanically supported compartment greatly contributes to the stability of the genome, hence allowing eukaryotes to manage ~1000-fold larger genomes than prokaryotes. Indeed, not only the size, but secondly also the structure of the genome dramatically changed from (predominantly) bacterial operons containing multiple open reading frames that are transcribed into polycistronic mRNAs to the typical intron/exon structure of modern eukaryotic genes, which are first transcribed into precursor mRNAs and eventually spliced into mature monocistronic mRNAs (Alberts et al., 2002). Importantly, the spatiotemporal separation of transcription (and concomitantly RNA processing) from translation was an inevitable prerequisite for (alternative) mRNA splicing to evolve and manifest itself as a mechanism to greatly enhance the coding potential of eukaryotic genomes, since the translation of un- or incompletely spliced transcripts, which could give rise to non-functional proteins or proteins with dominant-negative effects, is thereby elegantly prevented (as e.g. also discussed in Görlich and Kutay, 1999). In this regard, it is also noteworthy that individual exons in general encode for independent protein domains

and that recombination of previously unlinked exons via exon shuffling thus greatly favored the evolution of multi-domain proteins with diverse functions and specificities (Gilbert, 1978). Thirdly, the possibility to control the access of regulatory molecules such as transcription factors to the genetic information allows to fine-tune gene expression, e.g. in response to specific intra- or extracellular signals. Finally, a general benefit of compartmentalization is the local concentration of specific factors, thus increasing the efficiency of the catalytic reactions conducted in a given cellular organelle. Taken together, these new possibilities played a pivotal role in the evolution of eukaryotes into complex multicellular organisms.

1.1 The Fundamental Aspects of Nucleocytoplasmic Exchange

Evidently, the information stored and processed in the nucleus has to reach the cytoplasm to be read and interpreted. Therefore, mature RNA species (especially protein-coding mRNAs) and ribonucleoproteins (RNPs; e.g. fully assembled ribosomal subunits) have to be exported to the cytoplasm, whereas all nuclear proteins such as histones, transcription factors or the components of the DNA replication machinery need to be imported into the nucleus. Indeed, nucleocytoplasmic transport is characterized by an immense bidirectional mass flux (estimated to amount to ≈10-20 MDa·NPC^{-1}·s^{-1}; Ribbeck and Görlich, 2001) and demands considerable cellular resources. Taken together, the complete nuclear transport machinery comprises (i) nuclear pore complexes (NPCs), which are large proteinaceous gateways allowing for nucleocytoplasmic exchange, (ii) nuclear transport receptors (NTRs) dedicated to chaperone selected molecules into or out of the nucleus and (iii) the RanGTPase system, which feeds in metabolic energy, thereby bestowing directionality to the transport processes.

All exchange of macromolecules between nucleus and cytoplasm proceeds solely through **NPCs** (Feldherr et al., 1984), which perforate the nuclear envelope (Watson, 1954). NPCs are large, structurally conserved assemblies built from multiple copies of approximately 30 different proteins (Rout et al., 2000; Cronshaw et al., 2002) called nucleoporins (Nups). They comprise a rigid core scaffold with eightfold rotational symmetry (Wischnitzer, 1958; Gall, 1967) and a central channel equipped with a permeability barrier.

Importantly, the permeability barrier allows two modes of passage through the nuclear pore: (i) passive diffusion, which is efficient only for small molecules below a size limit of 40 kDa (Harding and Feldherr, 1958; Bonner, 1975; Mohr et al., 2009), and (ii) facilitated transport of even larger cargo molecules (reviewed e.g. in Görlich and Kutay, 1999; Fried and Kutay, 2003). In order to handle the vast nucleocytoplasmic mass fluxes, each NPC accommodates for ~1,000 facilitated translocation events per second, turning over the

equivalent of its own mass (~125kDa in higher eukaryotes) almost every second (Ribbeck and Görlich, 2001).

Facilitated transport relies on **NTRs** (Moore and Blobel, 1992; Görlich et al., 1994; Imamoto et al., 1995; Görlich et al., 1995a; 1995b; Pollard et al., 1996; Görlich, 1997; Fornerod et al., 1997a), which shuttle between the nucleus and cytoplasm. NTRs bind to specific import (e.g. the classical nuclear localization signal (NLS) of the simian virus 40 large T-antigen, Kalderon et al., 1984) or export (e.g. the classical leucine-rich nuclear export signals, see also Güttler et al., 2010) signals on the cargo molecules and mediate their translocation through the nuclear pore.

Most NTRs belong to the Impβ superfamily and hence share a number of features such as their relatively large size (typically 90-150kDa), their overall negative charge and their ability to bind the small guanine nucleotide-binding protein Ran (Görlich et al., 1997; Fornerod et al., 1997a). Indeed, Impβ-like NTRs are even build up out of the same basic structural units (reviewed e.g. in Cook et al., 2007; Güttler and Görlich, 2011), so called HEAT repeats (for *h*untingtin, *e*longation factor 3, the PR65/A subunit of protein phosphatase 2*A* and the lipid kinase *T*OR, which were the first proteins identified to posses this structural motif). One HEAT repeat unit is comprised of two antiparallel alpha helices connected by a short linker. Multiple HEAT repeats are then stacked in a tandemly arranged fashion into superhelical solenoids, giving Impβ-like NTRs their characteristic shape.

A complete translocation round for Impβ-type NTRs (in the following referred to as NTRs for simplicity) comprehends six defined steps (see also Figure 1.1): (i) recognition of cargoes by cognate NTRs, (ii) docking of the NTR•cargo complexes to the NPC and penetration into the permeability barrier, (iii) the actual translocation through the permeability barrier, (iv) exit from the permeability barrier, (v) NTR•cargo complex disassembly and (vi) retrieval of the NTRs to the starting compartment (strictly seen including another docking, translocation and exiting step). According to the directionality of the transport routes they participate in, NTRs can be classified as either Importins (Imps) or Exportins (Exps), whereas only few are known to function as both an Importin or Exportin (see Table 1.1).

Table 1.1. *Nuclear transport receptors of the Impβ-family and their cargoes.*[1]

– Importins –		
NTR	Adaptor or co-receptor	Selected cargoes
Importin β (Impβ-1)	Importinα	Ribosomal proteins HIV Rev, HIV Tat Histones Classical NLS-containing cargoes
	Snurportin 1	m_3G-capped U-snRNPs
	XRIPα	Replication protein A
	Importin 7	Histone H1

Transportin 1+2 (Trn, Impβ-2)		hnRNP proteins (M9-NLS) Ribosomal proteins
Transportin 1+2 (Trn, Impβ-2)		TAP Histones c-FOS SRP19
Transportin SR 1+2 (TrnSR, Trn3)		SR proteins tRNA
Importin 4		Ribosomal proteins Histones
Importin 5		Ribosomal proteins Histones
Importin 7	Importin β	Ribosomal proteins Histone H1 ERK2, SMAD3, MEK1
Importin 8		SRP19 Argonaute proteins
Importin 9		Histones Ribosomal proteins
Importin 11		UbcM2 Ribosomal protein L12
– Exportins –		
NTR	Adaptor or co-receptor	Selected cargoes
Crm1 (Exportin 1)	HIV Rev PHAX	Leu-rich NES cargoes RRE-containing RNAs m^7G-capped U snRNAs Snurportin 1
CAS (Exportin 2)		Importin αs
Exp-t		tRNA
Exportin 5	aa-tRNA dsRNA	eEF1A dsRNA-binding proteins pre-miRNAs
Exportin 6		Actin•profilin complexes
Exportin 7		p50 RhoGAP, 14-3-3σ
– Bi-directionally operating NTRs –		
NTR	Adaptor or co-receptor	Selected cargoes
Importin 13		UBC9, MGN/Y14 (import) eIF1A (export)
Exportin 4		eIF5A (export) SMAD3 (export) Sox2, SRY (import)

[1] Compiled based on (Görlich and Kutay, 1999; Fried and Kutay, 2003; Güttler and Görlich, 2011).

Notably, Impβ does not directly bind to many of its import cargoes, hence undergoing a slightly more complicated translocation cycle including an additional component (for a detailed review, see Görlich and Kutay, 1999). The latter is the import adaptor Impα,

which recognizes the classical NLSs of a broad range of cargo molecules in complex with Impβ (Görlich et al., 1994; 1995a; 1995b). A separate high-affinity signal sequence on Impα, the importin β-binding domain (IBB), mediates the interaction with Impβ (Görlich et al., 1996a).

Imp-α proteins are build from armadrillo (ARM) motifs (Görlich and Kutay, 1999), which are structurally related to the HEAT repeats of *bona fide* NTRs of the Imp-β subfamily (Andrade et al., 2001). However, even though Imp-α is likely to have a higher 'solubility' in the NPC permeability barrier than truly inert proteins (see Section 1.3), it is nevertheless a poor mediator of facilitated translocation independent of Imp-β (Görlich et al., 1995a; 1995b).

An important property of NTRs is their regulation by **Ran**. Like related small GTP-binding proteins, Ran functions as a molecular switch residing either in an ON or OFF state, depending on whether GTP or GDP is bound, respectively. The change of states is accompanied by large conformational changes in Ran (see also Figure 2.4 and Scheffzek et al., 1995; Vetter et al., 1999; Partridge and Schwartz, 2009), which can be transmitted to interacting NTRs.

As the intrinsic nucleotide hydrolysis capability of Ran is very low, the molecule cannot switch states on its own, but rather requires the help of the GTPase-activating protein RanGAP (in conjunction with the Ran binding proteins RanBP1 or RanBP2/Nup358) to hydrolyze the bound GTP (Bischoff et al., 1994; Bischoff and Görlich, 1997) and the guanine nucleotide exchange factor Rcc1 to exchange GDP for GTP (Bischoff and Ponstingl, 1991a).
Due to the restriction of RanGAP, RanBP1 and RanBP2/Nup358 to the cytoplasm or cytoplasmic face of the NPC and the chromatin-association of Rcc1, a steep RanGTP gradient is establish across the nuclear envelope, with high nuclear and low cytoplasmic concentrations (Görlich et al., 1996b; Izaurralde et al., 1997). Thus, Ran can convey information about the identity of the compartment to the shuttling NTRs, which *per se* can traverse the nuclear pore (even in a cargo-bound state) in either direction in an energy-independent manner (Kose et al., 1997; Schwoebel et al., 1998; Nachury and Weis, 1999; Ribbeck et al., 1999).

In molecular terms, Importins and Exportins respond diametrically opposite to binding of RanGTP (Figure 1.1). For Importins, which form dimeric import complexes with their cargoes in the cytoplasm, RanGTP binding causes the dissociation of the import complex and release of the cargo in the nucleus (Rexach and Blobel, 1995; Görlich et al., 1996b). Exportins however can only bind their cargoes with affinity when engaging in a trimeric export complex with RanGTP (Fornerod et al., 1997a; Kutay et al., 1997a). RanGAP- and RanBP1/2-aided nucleotide hydrolysis in the cytoplasm in turn leads to export complex disassembly and cargo release. Thus, the chemical potential of the RanGTP gradient is the driving force of directional nucleocytoplasmic transport

(Izaurralde et al., 1997; Görlich et al., 2003), moreover allowing the accumulation of transport substrates against gradients of chemical activity.

Note however that active nuclear transport would soon deteriorate the RanGTP gradient over time if the RanGDP that accumulates in the cytoplasm due to export complex disassembly and retrieval of Importins were not re-imported into the nucleus and converted into RanGTP again. This duty is fulfilled by NTF2 (Ribbeck et al., 1998; Smith et al., 1998), a dedicated import receptor for RanGDP (Stewart et al., 1998) importantly not belonging to the Impβ superfamily (Bullock et al., 1996). Hence in this case, cargo release can be independent of RanGTP binding, an essential requirement to abstain from futile cycles of Ran shuttling. Instead, RanGDP release is believed to rely on the concerted action of Rcc1 and Impβ-like NTRs (Ribbeck et al., 1998; Smith et al., 1998).

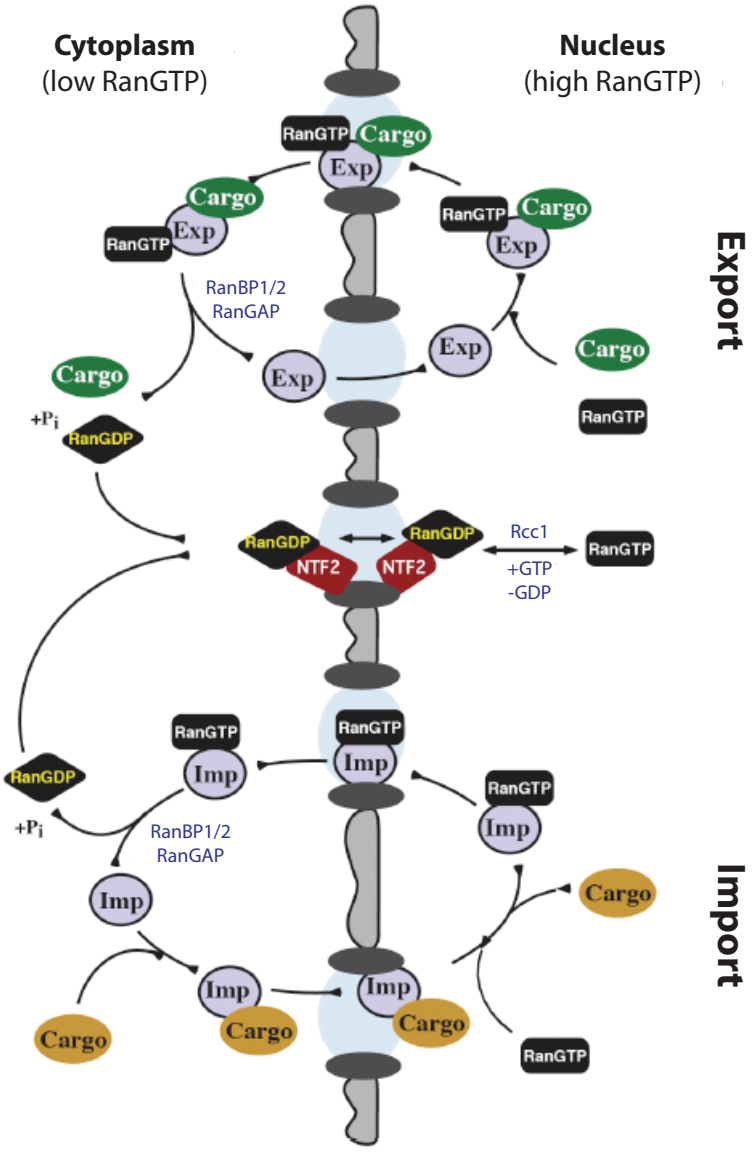

Figure 1.1. Schematic overview of the nuclear export and import cycles and their regulation by the RanGTPase system. Adapted from (Görlich and Kutay, 1999). 'Exp' denotes Exportin and 'Imp' stands for Importin. See main text for further details.

In summary, the NPC permeability barrier efficiently restricts the diffusion of small proteins (i.e. below >5nm in diameter) into and out of the nucleus, yet paradoxically, it requires the help of large NTRs even for similar sized cargoes to surmount this size barrier in a reasonable time. In turn, how such a barrier operates and what the physical nature of the NPC permeability barrier is, remain the major unsolved and highly debated questions in the field of nucleocytolasmic transport, and essentially motivated this study. In the following, the molecular architecture of the NPC (Section 1.2) and current models of NPC permeability barrier function (Section 1.3) will be introduced in more detail to summarize the collective proceedings of the field and to deduce the specific aims of this work.

1.2 The Nuclear Pore Complex

The concepts of nucleocytoplasmic transport differ in many aspects from other intracellular transport pathways, such as protein import into the ER, mitochondria, chloroplasts or peroxisomes (to name a few). Maybe most importantly, small molecules and metabolites, as well as ions, can freely exchange between nucleus and cytoplasm, whereas great care is taken so that the specific, fine-tuned biochemical milieu of other compartments is not disturbed due to material exchange with the cytoplasm. This is because molecules do not have to be transported across a lipid bilayer to enter or exit the nucleus. Rather, the outer and inner membranes of the NE are fused at the sites where NPCs reside, leaving an aqueous passage that is however guarded by the NPC permeability barrier. Hence, NPCs function both as 'grommets' that reinforce the pores in the NE and selective gates for bidirectional nucleocytoplasmic exchange.

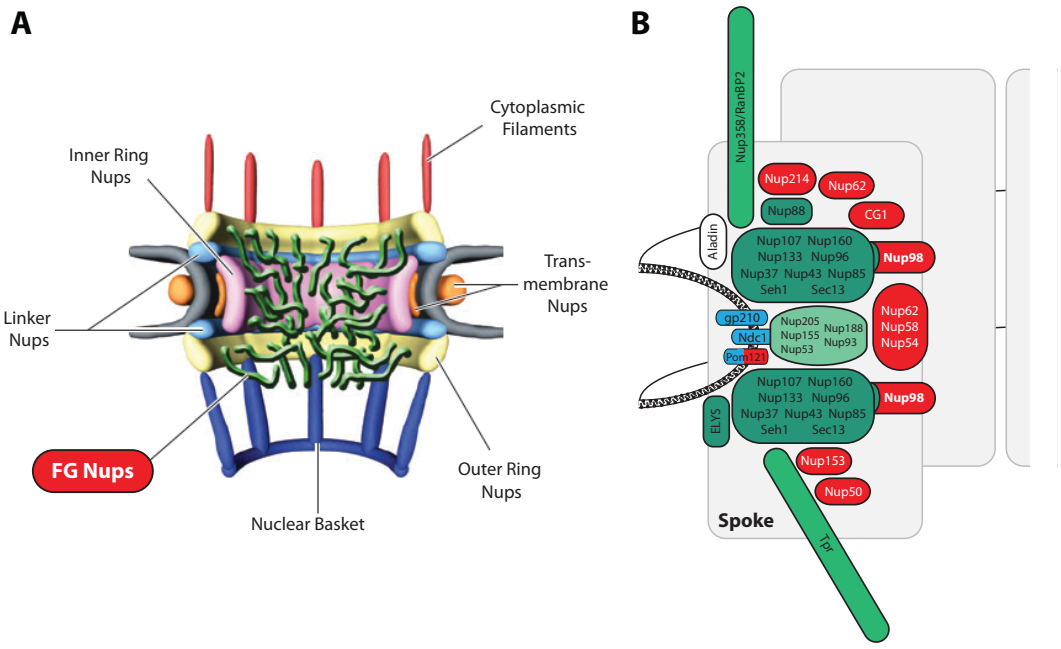

Figure 1.2. The architecture of the nuclear pore complex (NPC). (A) Schematic drawing of the NPC (adapted from Grossman et al., 2012), illustrating how the outer ring, linker, inner ring and transmembrane Nups form the core NPC scaffold. The permeability barrier is constituted by the FG Nups filling the central channel of the NPC. Extending into the cytoplasm are the cytoplasmic filaments. A basket-like structure is found at the nuclear face of the NPC. (B) The basic symmetry unit of the NPC is a 'spoke'. Shown here are the Nup subcomplex that comprise a single spoke. Please note that eight spokes align in the equatorial plane of the nuclear envelope to form the multiple coaxial rings of the core scaffold. See main text for details, especially on the Nup subcomplexes.

1.2.1 The NPC is a highly modular macromolecular assembly.

Different parts of the highly modular NPC structure serve these two purposes (Figure 1.2). The core scaffold of the NPC, which comprises two outer and two inner coaxial rings, forms the grommet-like structure (reviewed e.g. in Grossman et al., 2012). It is anchored to the NE via integral membrane Nups that interact with components of the inner ring (Onischenko et al., 2009). Extending from the outer rings into the cytoplasm and nucleus are eight cytoplasmic filaments and a nuclear basket-like structure, respectively (Jarnik and Aebi, 1991; Goldberg and Allen, 1992). The inner walls of the coaxial rings are lined with Nups containing so-called phenylalanine-glycine (FG) repeat domains, which are thought to form the permeability barrier (see below for details).

Electron microscopy studies of *Xenopus* (Unwin and Milligan, 1982; Hinshaw et al., 1992; Akey and Radermacher, 1993; Akey, 1995; Stoffler et al., 2003; Frenkiel-Krispin et al., 2010), *Dictyostelium* (Beck et al., 2004; 2007), yeast (Yang et al., 1998; Kiseleva et al., 2004; Alber et al., 2007) and human (Maimon et al., 2012) NPCs showed that their general architecture is well conserved. The reported size estimates of the NPC range from ~65MDa to ~125MDa, depending on both the species analyzed and the methods used (Reichelt et al., 1990; Rout and Blobel, 1993).

The basic symmetry unit of the NPC is referred to as a spoke, which is constituted by distinct biochemically defined sub-complexes of either (i) transmembrane, (ii) core scaffold or (iii) FG repeat domain Nups (Figure 1.2). Eight spokes align in an equatorial plane to assemble into the multiple coaxial rings of the core scaffold, explaining the characteristic eightfold rotational symmetry already observed in the first electron micrographs of NPCs (Gall, 1967). Moreover, this organization also requires that each Nup is present in single or multiple copies of eight. Taking into consideration that NPCs are comprised of ~30 distinct Nups (Rout et al., 2000; Cronshaw et al., 2002), at least ~240 protein molecules are required to build the complete structure. The exact copy numbers of the different Nups are still largely under debate, but it is assumed that NPCs are composed of as many as ~700 individual molecules (Rout et al., 2000). Despite this large number of contributing parts, the symmetry constraints, the organization of most Nups into sub-complexes and the findings that Nups are only made up of limited domain topologies greatly reduce the overall (structural) complexity of the NPC.

1.2.2 Defined sub-complexes are the major building blocks of higher order NPC structure.

In context of the NPC, individual sub-complexes are defined based on the ability of a subset of Nups to stably interact with each other, even when the nuclear envelope breaks down during open mitosis. Indeed, NPCs reassemble from the fragmented sub-complexes at the end of mitosis (reviewed in Dangelo and Hetzer, 2008), highlighting their importance as the major building blocks of higher order structural elements (i.e. single spokes). Notably, based on mainly biochemical analysis of homologous components, the same sub-complexes can also be identified in eukaryotes undergoing closed mitosis (e.g. yeast). Historically, Nups are named after their molecular weight, which might however vary for homologous proteins from distantly related organisms. In the following, the mammalian nomenclature is used when referring to specific Nups, unless explicitly stated otherwise. For comparison, Table 1.2 gives an overview of the known Nups identified in various Eukaryotes. Notably, recent progress in genome sequencing and improvement of algorithms for homology detection suggest that the different sub-complexes are evolutionary well conserved (Mans et al., 2004; Bapteste et al., 2005; Neumann et al., 2010).

Table 1.2. *Vertebrate nucleoporins and their (known) homologs in selected species.*

Localization	Vertebrates[1]	Yeast[1] (*S. cerevisae*)	*Tetrahymena thermophila*[2]	*Trypanosoma brucei*[3]
Cytoplasmic fibrils	Nup88	Nup82		
	Nup214	Nup159	Nup192?	
	Nup358/RanBP2	-		Nup308?
Transmembrane	Ndc1	Ndc1		
	Pom121	-		
	gp210	Pom152	pp210	
Outer Ring	Nup85	Nup85		
	Nup96	Nup145C	Nup96	Nup158
	Nup107	Nup84		Nup82/Nup89
	Nup133	Nup133		Nup109/Nup132
	Nup160	Nup120		Nup109/Nup132
	Sec13	Sec13	Sec13	Sec13
	Seh1	Seh1	Seh1	
	Nup37	-		
	Nup43	-		
	ELYS	-		
	Aladin	-		Nup48
Inner Ring	Nup53	Nup53		
	Nup93	Nic96	Nup93	Nup96

Inner Ring	Nup155	Nup157/Nup170	Nup155	Nup144
	Nup188	Nup188		Nup181/Nup225
	Nup205	Nup192	Nup199?	Nup181/Nup225
Central channel	Nup54	Nup57	Nup54	Nup53a/Nup53b?
	Nup58	Nup49		Nup58?
	Nup62	Nsp1		Nup62?
	Nup98	Nup100/Nup116/ Nup145N	MacNup98A/B MicNup98A/B	Nup158
	CG1	Nup42		
Nuclear Basket	Tpr	Mlp1/Mlp2		Nup92/Nup110
	Nup50	Nup2	Nup50	
	Nup153	Nup1		

[1] For a comparison of vertebrate and yeast Nups, see e.g. (Grossman et al., 2012).
[2] See (Malone et al., 2008; Iwamoto et al., 2009) and Table 1.3.
[3] See (Degrasse et al., 2009).

The major constituent of the outer ring is the heptameric **Y complex**, named after its characteristic Y-shape observed in electron micrographs (Siniossoglou et al., 2000; Lutzmann et al., 2002). It comprises the major components Nup85, Nup96, Nup107, Nup133, Nup160, Sec13 and Seh1, plus the additional proteins Nup37, Nup43 and ELYS in many eukaryotes (excluding for example the yeast *Saccharomyces cerevisiae*, which nevertheless is a well established model organism in the field). Associated with the Y complex is the FG repeat domain-containing nucleoporin (FG Nup) **Nup98**, which interacts directly with Nup96. Importantly, Nup98 is considered to be the major constituent of the NPC permeability barrier (see also Section 1.3 and (Laurell et al., 2011; Hülsmann et al., 2012). Interestingly, Nup98 is post-translationally O-glycosylated in vertebrates such as *Xenopus laevis* and *Homo sapiens* (Powers et al., 1995; Radu et al., 1995).

The inner ring is formed by the **Nup93-Nup205 sub-complex**, which mainly consists of Nup93, Nup188 and Nup205 (Grandi et al., 1997; Miller et al., 2000), but in some models also contains Nup53 and Nup155 (Hawryluk-Gara et al., 2005), through which interaction the entire sub-complex is linked to the transmembrane Nups Ndc1 (Mansfeld et al., 2006; Stavru et al., 2006), Pom121 (Hallberg et al., 1993) and gp210 (Gerace et al., 1982). The Nup93-Nup205 sub-complex is not as defined as the well-studied Y complex, mainly because the interactions between the two constituting parts are well documented, but not as stable as the interactions within the parts. Thus, in *S. cerevisiae* for example, the Nup35 and Nup155 homologs are sometimes considered to form an independent sub-complex. Attached to the Nup93-Nup205 sub-complex is the **Nup62 sub-complex** (Finlay et al., 1991), which is hence located to the center of the NPC. It comprises the FG nucleoporins Nup54, Nup58 and Nup62. Like Nup98, the latter is O-glycosylated in vertebrates (Davis and Blobel, 1987).

The cytoplasmic filaments are comprised of the **Nup214-Nup88 sub-complex** (Kraemer et al., 1994; Bastos et al., 1997; Fornerod et al., 1997b), and also contain