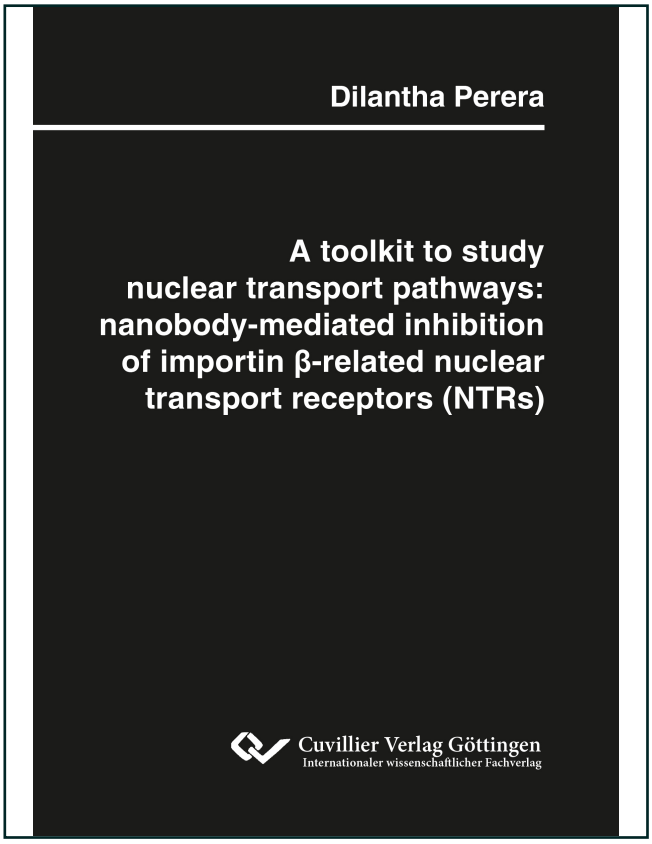
Dilantha Perera (Autor)

A toolkit to study nuclear transport pathways: nanobody-mediated inhibition of importin β-related nuclear transport receptors (NTRs)



https://cuvillier.de/de/shop/publications/8870

1 Introduction

1.1 Compartmentalization of eukaryotic cells

The main distinction between eukaryotes and prokaryotes, is that eukaryotic cells harbor numerous membrane-bound organelles, which allows for the separation of cellular material and processes by means of a physical barrier. The most prevalent example of this segregation can be observed when considering the two main compartments of eukaryotic cells, i.e., the nucleus and the cytoplasm.

The nucleus is the largest organelle in the cell and is enclosed by two concentric lipid bilayers, forming the nuclear envelope. The nucleus hosts the genomic DNA of the cell and protects it from physical and metabolic damage. As a result, the nucleus is also the site of numerous cellular processes that involve genomic DNA. This includes, but is not limited to, DNA replication and repair, transcription, and pre-mRNA splicing. These processes, along with their associated protein machinery, are also confined to the nucleus. The nucleus also hosts a sub-compartment, called the nucleolus, which is the site of ribosomal RNA synthesis and pre-ribosomal subunit assembly.

The cytoplasm plays host to cellular processes such as translation, and consists of various membrane bound organelles, such as the Endoplasmic reticulum (ER) and the Golgi apparatus, each with their own functions and protein constituency. These organelles are surrounded by an aqueous matrix termed the cytosol. The cytosol itself consists of ions, macromolecules, and other elements such as ribosomes and the cytoskeleton.

The restriction of certain metabolic processes to either the nucleus or the cytoplasm confers several advantages to eukaryotic cells; most notably, it allows for a robust regulation of gene expression. However, the two compartments are not completely isolated as they must still be able to exchange materials in order to link the different cellular processes (e.g. transcription to translation) (Görlich and Kutay, 1999). In fact, more than a million macromolecules can be actively transported between the nucleus and the cytoplasm per second (Ribbeck and Görlich, 2001). This nucleocytoplasmic transport is achieved by means of a selective physical barrier, i.e., the nuclear pore complex, working in conjunction with proteins that facilitate transport through the aforementioned barrier – nuclear transport receptors.

1.2 Nuclear pore complex

The nuclear pore complex (NPC) is a proteinaceous channel embedded in the nuclear envelope where the inner and outer membranes fuse (Watson, 1954). It functions as the ‘gateway’ that connects the nucleus to the cytoplasm. With a molecular weight of ≈ 125 MDa (Reichelt et al., 1990) in vertebrates and an outer diameter of ≈ 120 nm (Lin and Hoelz, 2019), these behemoths are amongst the largest protein complexes in eukaryotic cells. The number of NPCs per cell

depends on the type of cell and species in question – in HeLa cells, there are about 3,000 (Maul et al., 1972).

1.2.1 Architecture of the NPC

NPCs are made up of multiple copies of about 30 different proteins, termed nucleoporins (Nups) (Hoelz et al., 2011; Knockenhauer and Schwartz, 2016). Each Nup is present in NPCs in multiples of eight and the total number of Nups per NPC adds up to ≈ 1000 polypeptides (Cronshaw et al., 2002; Ori et al., 2014). Nucleoporins themselves, assemble into Nup sub-complexes and into three stacked rings (Figure 1.1) with an eight-fold rotational symmetry (Gall, 1967; Kim et al., 2018).

The NPC is anchored to the nuclear envelope by the proteins NDC1 and POM121, which form the luminal ring of the pore (Zhang et al., 2020). The luminal ring, in turn, serves as the attachment points for the inner ring (Kosinski et al., 2016; Lin et al., 2016). The inner ring scaffold is made up of the Nup62 complex which is anchored to the Nup93 complex (Chug et al., 2015). The outer rings of the NPC are built on the Y-complex scaffold (Stuwe et al., 2015b) which serves as a foundation for the attachment of Nup98, and the asymmetrically distributed components of the outer rings (nuclear and cytoplasmic). The nuclear outer ring comprises of the TPR nuclear basket, Nup50, and Nup153 (Cordes et al., 1993; Krull et al., 2004). The cytoplasmic outer ring is composed of anchored cytoplasmic filaments such as Nup214, Nup358, and Nup98 (Huang et al., 2020).

The NPC scaffold surrounds a large aqueous channel wherein lies the permeability barrier. This dense barrier is formed by intrinsically disordered phenylalanine-glycine (FG) repeat domains of some of the scaffold-anchored nucleoporins (Figure 1.1) that protrude into the central channel of the NPC. The properties of these FG repeats are what confers the selectivity of the NPC permeability barrier (Frey et al., 2006; Frey and Görlich, 2007; Hülsmann et al., 2012).

1.3 Permeability barrier of the NPC

The selective parameters of the permeability barrier are primarily the size and surface properties of the macromolecules being transported (Frey et al., 2018), i.e., the propensity of the protein surface residues to interact with the FG repeat containing domains (Allen et al., 2001; Rexach and Blobel, 1995). It permits the passive diffusion of small molecules but generally hinders the diffusion of larger proteins with a molecular weight > 40 KDa or a Stocks radius > 2.5 nm (Mohr et al., 2009).

1.3.1 FG repeat domains and FG-Nups

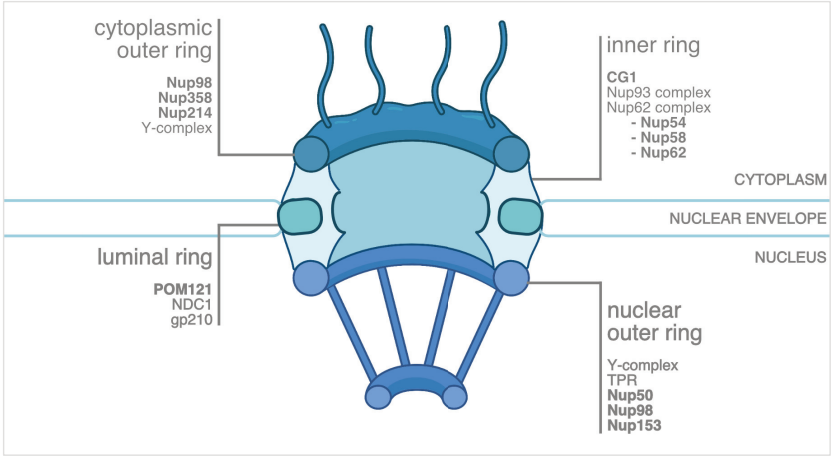


Figure 1.1: Illustration of a vertebrate nuclear pore complex (created with biorender.com)

NPC is depicted as three stacked rings embedded in the nuclear envelope. Nups are organized into the different modules described in the text. Not all Nups are shown. Not to scale. FG-Nups are represented in bold font.

Nups containing the FG repeat domains (FG repeats) that make up the NPC permeability barrier were first identified in *S. cerevisiae* (Davis and Fink, 1990; Nehrbass et al., 1990) and are called FG-Nups. FG-Nups are highly conserved throughout species (Terry and Wente, 2009) (Table 1.1), critical for cell viability (Strawn et al., 2004), and are essential to NPC selectivity (Frey and Görlich, 2007; Hülsmann et al., 2012; Patel et al., 2007). FG repeats are generally intrinsically disordered and comprise of up to 50 individual FG repeat units. These units refer to hydrophobic sequences, such as FG, FxFG or PAFG, embedded in a precise spacer sequence context (Denning and Rexach, 2007; Frey and Görlich, 2009; Labokha et al., 2013). Several of the Nups described earlier are considered to be FG-Nups and are anchored to the NPC scaffold via structured terminal domains, where their FG domains protrude into the central channel of the NPC and form the permeability barrier (reviewed in Schmidt and Görlich, 2016). Altogether, about 5000 FG repeats have been estimated to be present in each NPC (Ori et al., 2013).

As alluded to earlier, apart from small passively diffusing molecules, large macromolecules are routinely, and rapidly, transported to and from the nucleus (Ribbeck and Görlich, 2001). This high rate of facilitated active transport through the nuclear pore complex is facilitated by nuclear transport receptors (NTRs) which transport molecules in a highly parallel and bidirectional manner (reviewed in Görlich and Kutay, 1999). The surface of NTRs can interact with FG repeats at multiple sites (Bayliss et al., 1999; Bayliss et al., 2002, Isgro and Schulten, 2005; Port et al., 2015). The exact means by which FG repeats selectively permit NTR-mediated transport through the NPC had been a matter of intense debate.

Table 1.1: Vertebrate FG-Nups and their homologues in yeast (*S. cerevisae*)

NPC localization	Vertebrates	*S. cerevisae*
Inner ring	Nup54	Nup57
	Nup58	Nup49
	Nup62 complex	Nsp1p complex
	CG1	Nup42
Nuclear outer ring	Nup98	Nup145N
	Nup153	NUP1p
	Nup50	NUP2p
Cytoplasmic outer ring	Nup214	Nup159p
	Nup98	Nup116
	Nup358	n.d*
Luminal ring	POM121	n.d*

*n.d = not determined

1.3.2 Models of NPC Transport selectivity

Numerous models had been proposed to explain the mechanism of transport through the NPC; this included the 'virtual gating' model, 'reversible collapse model', the 'Kap-centric' model, the 'forest model', and the 'ring-cycle' model (reviewed in Schmidt and Görlich, 2016). However, most of these models attempted to explain how NTRs traversed through the channel without adequately addressing the key aspect of NPC transport selectivity, i.e., how does the NPC restrict the free flow of some molecules more strongly than that of others?

One of the key developments in the field came about due to the observation that, even though NTRs facilitated faster transport through the NPC, molecules overall - including NTRs - moved slower than anticipated while passing through the pore, i.e., had the NPC channel been filled with aqueous medium (Ribbeck and Görlich, 2001). This implied the existence of a selectively permeable barrier and led to the 'selective phase' model (Ribbeck and Görlich, 2001) (Frey and Görlich, 2007) (Frey and Görlich, 2009) (Figure 1.2). The model considers the NPC permeability barrier as a 3D meshwork (hydrogel) made of FG repeats, that are reversibly crosslinked by cohesive interactions. This meshwork is assumed to function as a 'molecular sieve', excluding inert molecules that are larger than the mesh size. NTRs can interact with the FG repeats at multiple sites on their surface (Isgro and Schulten, 2005; Port et al., 2015) and so, can open individual meshes and create a path through the hydrogel. When transporting inert cargoes, NTRs would have to shield them from the phase to ensure rapid transport. Failing to sufficiently shield cargoes would result in the translocating species not being able to fully partition into the permeability barrier and would likely lead to transport being aborted. The model also assumes that the FG hydrogel is adaptive and seals itself as the NTR makes its way through the pore, thus conserving its functional integrity. The 'selective phase' model is supported by observations that cohesive FG domains are critical to maintain NPC selectivity, and also by the fact that barrier-critical FG domains indeed spontaneously self-assemble into dense hydrogels that show NPC-like selectivity (Frey and Görlich, 2009; Labokha et al., 2013). Critically, it is the only model that adequately explains both, how NTRs facilitate transport through the NPC, and how FG domains form a selectively permeable barrier.

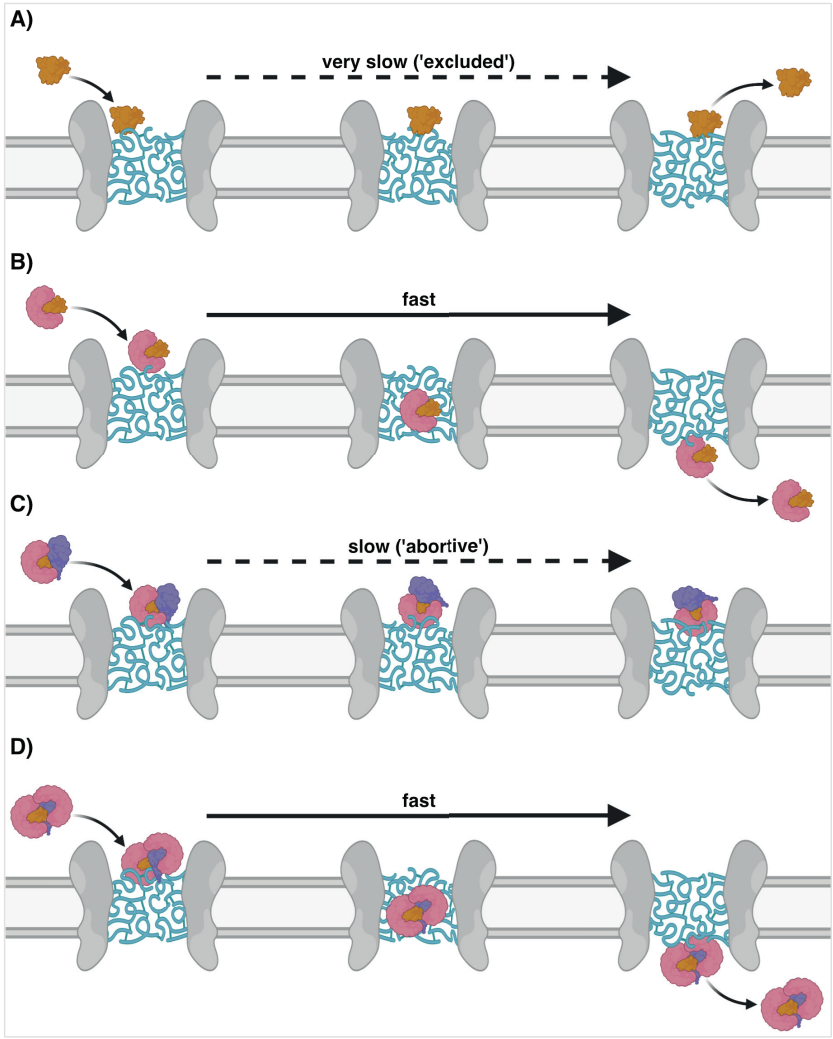


Figure 1.2: NPC passage according to the selective phase model (created with Biorender.com)

(A) Inert molecules (mustard) > 5 nm in diameter (or > 30 kDa) are excluded from the phase (blue). **(B)** NTRs (pink) facilitate the rapid passage of bound cargoes (mustard). **(C)** Cargoes that are not sufficiently shielded (purple) by NTRs are unable to completely transition into the phase and transport is eventually aborted. **(D)** Cargoes that are optimally shielded by NTRs are able to completely transition into the phase and rapidly traverse the through the phase. See text for details. Illustrated proteins are not representative of actual protein structure. Not to scale.

1.4 Nuclear transport receptors

Nuclear transport receptors (NTRs), also known as karyopherins, have surface properties that are highly favorable towards forming transient interactions with the FG-domains of the NPC permeability barrier (Bayliss et al., 1999; Chaillan-Huntington et al., 2000; Ribbeck and Görlich, 2001). The propensity of the NTR surface to interact with the FG-domains is mainly attributed to hydrophobic surface patches, though it has been observed that surface histidines, cysteines, and arginines might also contribute to the interaction (Frey et al., 2018). This enables NTRs to traverse the permeability barrier of the NPC in a facilitated manner. Consequently, NTRs can also bind and facilitate the transport of macromolecules (often referred to as cargoes), that are otherwise not permitted through the nuclear pore, into and out of the nucleus. Based on their function, NTRs can broadly be classified as either importins or exportins. Importins bind their cargoes in the cytoplasm and transport them, through the NPC, into the nucleus whereupon the import complex is disassembled, and the cargo is released. Exportins, in contrast, bind their cargoes in the nucleus and facilitate their transport through the NPC to the cytoplasm where the cargo is released upon export complex disassembly.

1.4.1 Characteristics of Importin β-like NTRs

The majority of nucleocytoplasmic transport is facilitated by members of the Importin β superfamily of NTRs (Görlich et al., 1997; Görlich and Kutay, 1999). These NTRs are rather large (95 – 145 kDa) and have been shown to share a similar domain organization, despite their poor sequence homology. Specifically, they all exhibit the same superhelical fold formed by about 20 HEAT repeats (Cingolani et al., 1999). HEAT repeats are made of two anti-parallel α-helices (termed A and B) that are connected by a short linker (Figure 1.3). The consecutive helices of the HEAT repeats that form the outer convex surface of the NTR, form hydrophobic grooves that promote the interaction with the NPC permeability barrier (Bayliss et al., 2002). Meanwhile, the helices that form the concave surface of the NTR serve as the interface for cargo and/or Ran (details to follow) binding. The repetitive organization within the NTR confers a degree of flexibility to its structure, allowing it to take on different conformations, from a closed ring to an open supercoil – rather like a molecular spring (Figure 1.3 C). This conformational flexibility aids in accommodating cargo binding and release (Conti et al., 2006).

The members of the Importin β superfamily are also characterized by their common biochemical properties, such as their acidic isoelectric point (pI = 4.6 – 6.0) and their affinity for phenyl-sepharose that can be seen as an FG mimic (Görlich and Kutay, 1999; Ribbeck and Görlich, 2002).

As with all other NTRs, the Importin β superfamily can also be divided into importins and exportins as described above. The directionality of facilitated transport is linked to another shared characteristic of Importin β-like NTRs – their ability to bind RanGTP (Görlich et al., 1997).

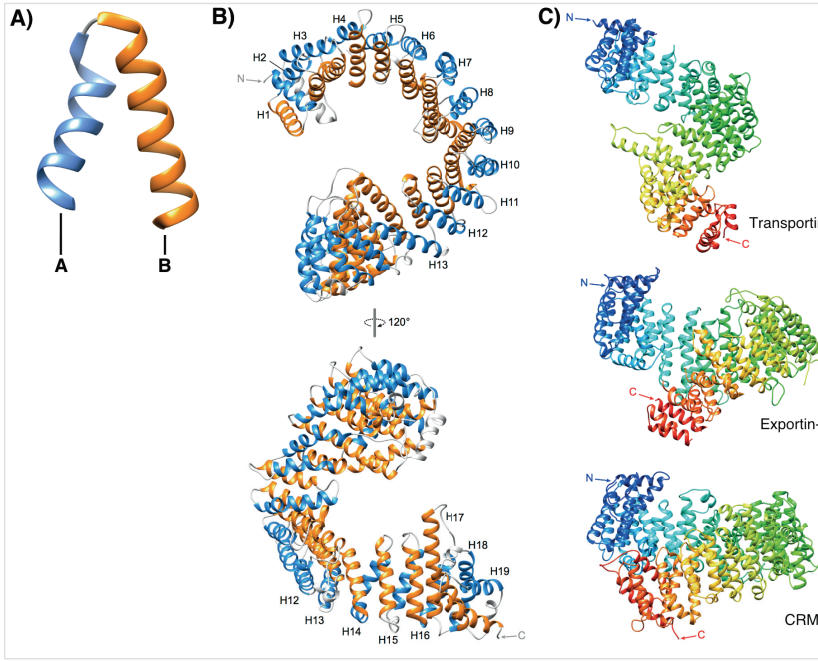


Figure 1.3: Architecture of Importin β-like NTRs
(Adapted with permission from Aksu, 2016)

Depicted as ribbon representations. **(A)** A single HEAT repeat with A and B helices highlighted in blue and orange, respectively. **(B)** HEAT repeat structure of Importin β (from the RanGTP• Importin β complex structure (PDB ID 2BKU, (Lee et al., 2005)) is shown in two different orientations. HEAT repeats are numbered according to (Lee et al., 2005). A and B helices are highlighted as in A, while loops and non-HEAT helices are colored in grey. N and C termini of the molecule are indicated. (C) The structures of Transportin (PDB ID 2OT8, (Cansizoglu and Chook, 2007)), Exportin-t (PDB ID 3ICQ, (Cook et al., 2009)) and CRM1 (PDB ID 3GJX, (Monecke et al., 2009)) are depicted to illustrate the conformational flexibility of NTRs. The transport receptors are shown with a color gradient from blue (N-terminus) to red (C-terminus).

1.4.2 RanGTP gradient and NTR transport directionality

Ran (Ras-related nuclear protein) is a small GTPase that belongs to the Ras superfamily (Bischoff and Ponstingl, 1991). Localized predominantly in the nucleus, it was the first nuclear transport factor to be identified (Melchior et al., 1993; Moore and Blobel, 1994). The GTPase activity of Ran means that it can be present in the cell in either a GTP-bound or a GDP-bound state. Importin β-like NTRs bind to RanGTP about 1000 times stronger than to RanGDP (Görlich et al., 1996b). The intrinsic activity of Ran to hydrolyze GTP to GDP, or to exchange GDP for GTP is rather slow. Instead, Ran depends on protein cofactors to promote these transitions.

The nucleotide exchange of RanGDP to RanGTP, is facilitated by the guanine nucleotide exchange factor (RanGEF), RCC1, which is itself bound to chromatin in the nucleus (Ohtsubo et al., 1989). This results in Ran being predominantly in the GTP-bound state when localized in the nucleus – a high concentration of RanGTP.

Meanwhile, the GTPase activity of Ran is promoted by the Ran GTPase activating protein RanGAP1 (Bischoff et al., 1994; Coutavas et al., 1993), and its cofactor, RanBP1 (Bischoff and Görlich, 1997), both of which are confined to the cytoplasm (Hopper et al., 1990; Melchior et al., 1993). This leads to cytoplasmic Ran assuming its GDP-bound state, or alternatively, a lower concentration of RanGTP in the cytoplasm. The consequence of this compartmentalization is the RanGTP gradient which determines the directionality of NTR transport (Görlich et al., 1996b; Izaurralde et al., 1997; Kalab et al., 2002) (Figure 1.4).

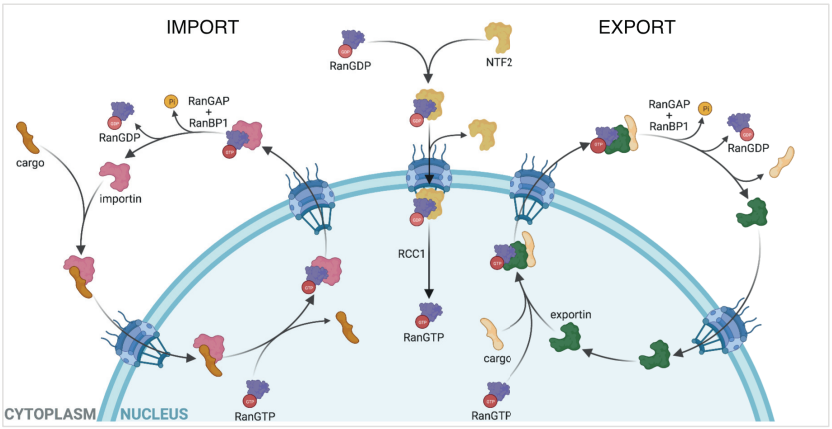


Figure 1.4: Nucleocytoplasmic transport machinery (created with Biorender.com)

A schematic overview of NTR facilitated nucleocytoplasmic transport coupled to the RanGTP gradient. Depicted from left to right - nuclear import, NTF2-mediated RanGTP gradient maintenance, and nuclear export. Illustrated proteins are not representative of actual protein structure. Not to scale.

Though both importins and exportins of the Importin β superfamily bind to RanGTP with a higher affinity than to RanGDP, the consequence of the interaction has antagonistic effects. In the case of importins, which bind their cargo in the cytoplasm and transport them through the NPC, RanGTP binding triggers cargo release in the nucleus (Görlich et al., 1996b; Rexach and Blobel, 1995). The RanGTP•importin complex then travels, back through the NPC, to the cytoplasm where importin is released following RanGTP hydrolysis. In contrast, exportins are able to bind stably to cargoes only in the presence of RanGTP (Kutay et al., 1997). This trimeric export complex then traverses the NPC and disassembles upon RanGTP hydrolysis in the cytoplasm. Free exportins are then able to transition back through the pores for subsequent rounds of export. It is also worth noting that some Importin β-like NTRs mediate the import of cargoes, while also being able to export another set of cargoes (Aksu et al., 2018; Gontan et al., 2009; Mingot et al., 2001). These NTRs are called bidirectional NTRs or 'biportins'.

Considering both importins and exportins export a Ran molecule per cycle, the nucleus would eventually be depleted of its Ran and the RanGTP gradient would break down. To avoid this predicament and maintain the Ran gradient, a dedicated

import pathway for RanGDP is mediated by another NTR – nuclear transport factor 2 (NTF2) (Ribbeck et al., 1998) (Figure 1.4).

1.4.3 RanGTP interaction with Importin β-like NTRs

To successfully utilize the RanGTP gradient for directed transport, NTRs have to distinguish between RanGTP and RanGDP (Figure 1.5) so as to only bind the GTP-bound state. Remarkably, the NTRs do not directly contact the bound GTP but rather rely on the structural differences between the two Ran states. A structural comparison between RanGTP and RanGDP reveal conformational differences in three main regions, referred to as the 'switch regions (I, II, and III)' (Scheffzek et al., 1995; Vetter et al., 1999). The NTRs can perceive the differences in switch I and II, and some, additionally interact with the basic patch of Ran that becomes exposed upon changes in the conformation of switch III and its C-terminal acidic DEDDDL motif.

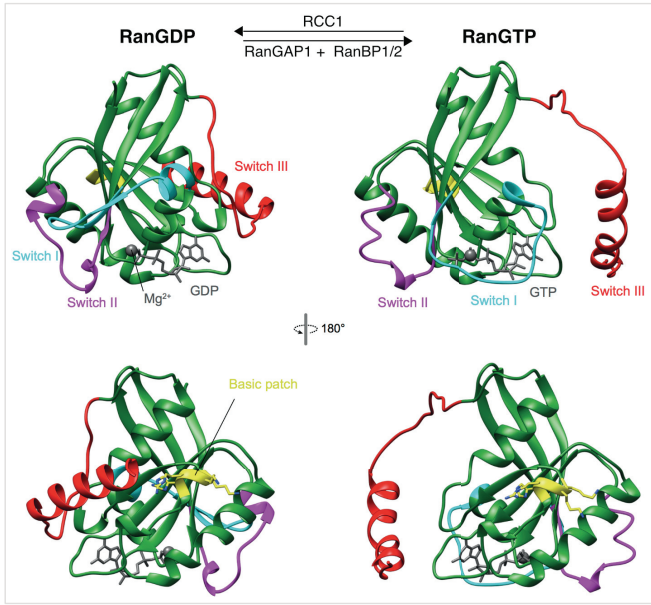


Figure 1.5: Structural comparison of RanGDP and RanGTP
(Adapted with permission from Aksu, 2016)

Illustrated as ribbon representations; the structures of Ran bound to GDP (left) and GTP (right) are shown in two different orientations. Mg^{2+} ions and the nucleotides are shown as spheres and sticks, respectively. The core of the protein (G domain) is colored in green, while the switches labelled and colored in cyan (switch I, residues 30-47), magenta (switch II, residues 65-80), red (switch II, residues 177-216) and yellow (basic patch, residues 139-142). Residues of the basic patch are shown in yellow sticks, while nitrogen atoms are in blue. The C-terminal DEDDDL acidic motif (residues 211-216) is not resolved in the crystal structures. See text for additional details. RanGDP and RanGTP structures have the PDBs ID 3GJ0 (Partridge and Schwartz, 2009) and 1RRP (Vetter et al., 1999), respectively.

As described earlier, the interaction of NTRs with RanGTP either triggers cargo release (in the case of importins), or promotes cargo binding (when considering

exportins). When considering the import pathway, this may be achieved by having the RanGTP-binding and cargo-binding sites of the importin partially overlap (Görlich et al., 1996b; Rexach and Blobel, 1995; Lee et al., 2005). Thus, binding of RanGTP or cargo to importins is mutually exclusive. Most importins have a higher affinity for RanGTP than the cargoes and so RanGTP will outcompete the cargo for the binding site, triggering cargo release. RanGTP binding to exportins, on the other hand, has a cooperative effect on cargo binding resulting in a stable trimeric export complex (Kutay et al., 1997; Kutay and Güttinger, 2005; Matsuura and Stewart, 2004).

1.4.4 Nuclear transport pathways

The Importin β superfamily of NTRs is the largest NTR class and consists about 20 members in vertebrates and 14 members in S. cerevisiae (Quan et al., 2008). Consequently, its members mediate the majority of nucleocytoplasmic transport pathways in the cell (Görlich and Kutay, 1999). As described earlier, these NTRs share many structural, functional, and biochemical similarities. However, they vary greatly when it comes to the size and diversity of their cargo repertoires. Significant progress has been made towards dissecting these NTR pathways over the past few decades (Table 1.2), particularly in the case of NTRs such as Importin β and CRM1. However, many NTR pathways remain elusive due to the complex nature of the network.

Importin pathways are responsible for delivering cytoplasmic proteins and macromolecules to the nucleus. In addition to transporting nuclear products to the cytoplasm, exportins also assume the crucial role of maintaining the 'compartmental identity' of the nucleus. As described earlier, the permeability barrier of the NPC is not an absolute barrier; small proteins can diffuse into the nucleus and some larger proteins can also passively cross the barrier given enough time. As a result, proteins that are supposed to function only in the cytoplasm (translation machinery), and proteins whose presence in the nucleus are highly regulated (e.g. transcription factors) can leak in and disrupt nuclear processes. Exportins, particularly CRM1, constantly export and deplete these cytoplasmic proteins from the nucleus (Kirli et al., 2015; Schwarzerová et al., 2019). Naturally, this further expands the network of NTR pathways.

It should be noted that there are other NTRs in addition to those of the Importin β superfamily. Examples include the aforementioned NTF2 (Ribbeck et al., 1998), Mex67p (Segref et al., 1997), and Mtr2p (Santos-Rosa et al., 1998; Yao et al., 2007). These proteins do not share structural similarities with Importin β.

Ascertaining the cargo repertoire of NTRs has long been a focus in the field of nuclear transport research. The identification of NTR-cargo pairs and patterns of NTR cargo recognition are key when it comes to dissecting an NTR pathway. Given the diversity of NTR cargo portfolios, it is worth expanding on how NTRs recognize their cargoes.