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Components and regulation of nuclear transport processes

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The spatial separation of DNA replication and gene transcription in the nucleus and protein translation in the cytoplasm is a uniform principle of eukaryotic cells. This compartmentalization imposes a requirement for a transport network of macromolecules to shuttle these components in and out of the nucleus. This nucleo-cytoplasmic transport of macromolecules is critical for both cell physiology and pathology. Consequently, investigating its regulation and disease-associated alterations can reveal novel therapeutic approaches to fight human diseases, such as cancer or viral infection. The characterization of the nuclear pore complex, the identification of transport signals and transport receptors, as well as the characterization of the Ran system (providing the energy source for efficient cargo transport) has greatly facilitated our understanding of the components, mechanisms and regulation of the nucleo-cytoplasmic transport of proteins in our cells. Here we review this knowledge with a specific emphasis on the selection of disease-relevant molecular targets for potential therapeutic intervention.

Introduction

The deregulation of the nuclear import and export machinery is a key marker of various diseases [1,2]. For example, many tumor suppressor transcription factors show cytoplasmic sequestration ablating their nuclear functions allowing, for example, uncontrolled cell division [3]. The aberrant localization of onco-proteins can also lead to their inadequate activation. Examples of aberrant nucleo-cytoplasmic shuttling that correlates with tumor formation, progression or resistance to treatment include p53, FOXO3a, p27, BRCA1, APC, nucleophosmin retinoblastoma, β-catenin, nuclear factor-κB (NF-κB), survivin and cyclin D1 [3–5]. Beyond cancer, many viruses including human HIV-1, influenza A, dengue, respiratory syncytial virus, rabies, Rift Valley fever virus and Venezuelan equine encephalitis virus rely on the transport of specific viral proteins into the host cell nucleus to perturb the anti-viral response. Therefore, inhibiting the nuclear trafficking of viral proteins has been proposed as a viable therapeutic strategy [6]. Over the last decade, the research community has

Abbreviations

CRM1, exportin1 or Xpo1, chromosome region maintenance 1; EM, electron microscopy; FG, phenylalanineglycine; FG-Nups, phenylalanineglycine nucleoporins; Imp-α, importin-α; Imp-β, importin-β; NE, nuclear envelope; NES, nuclear export signal; NF-κB, nuclear factor-κB; NLS, nuclear localization signal; NPC, nuclear pore complex; NTR, nuclear transport receptor; Nups, nucleoporins; POMs, Pore membrane proteins; PY-NLS, proline-tyrosine NLS.

acquired a critical mass of significant knowledge on the constituents of the nuclear transport machinery. The emerging picture, while intriguingly complex, suggests that there is an availability of a broad range of molecular targets enabling specific therapeutic interventions. The transport of cargo proteins is mediated by several distinct types of transport signals that are recognized by specific transport receptors or via a variety of adaptor proteins. These transport receptors can then interact with components of the nuclear pore complex (NPC) and with the Ras family GTPase Ran. Many of the steps within this process have the potential to be therapeutically targeted for innovative anti-cancer and anti-viral therapies.

The nuclear envelope and the nuclear pore complex

The nucleus is surrounded by an envelope composed of two phospholipidic membranes: an outer and an inner nuclear membrane that are 30 nm apart. The nuclear envelope (NE) provides a much stronger physical barrier than the single cordon of the plasma membrane. The outer nuclear membrane is continuous with the endoplasmic reticulum [7], whereas the inner nuclear membrane is associated with a network of intermediate filaments composed of lamin called the nuclear lamina. This acts as a site of attachment for chromosomes and as a shield for the nucleus [8]. The NE functions like a selectively permeable barrier allowing macromolecules to move between the nucleus and the cytoplasm via a gatekeeper, the NPC. The NPC is a huge protein complex that fuses the internal and external nuclear membrane to form an aqueous channel (Fig. 1). The NPC is cylindrical measuring 100–150 nm in diameter and 50–70 nm in thickness [9] and is broadly conserved in eukaryotes [10,11]. The molecular mass of the NPC is approximately 125 000 kDa and the number of NPCs per nucleus is highly variable among organisms. The average number of NPCs within a vertebrate cell is between 2000 and 5000 [12] and has been extensively studied by electron microscopy (EM). In particular the development of cryo-EM and cryo-electron tomography (cryo-ET) allowed structural preservation to be enhanced and purification steps to be minimized and as a consequence the detailed and artifact-free analysis of the NPC [12–15]. These EM-based studies revealed a highly modular and dynamic structure with a doughnut-shaped central core with an eightfold rotational symmetry [16]. A central channel is surrounded by three ring-like structures, namely the cytoplasmic ring, the central spoke ring and the nuclear ring. Attached to this core structure are eight protein filaments on the cytoplasmic side and eight protein filaments on the nuclear side that converge to a ring-like structure termed the nuclear basket.

The NPC is composed of 30 different proteins termed nucleoporins (Nups) [17] that are organized into several sub-complexes, each of which is present in multiple copies, resulting in approximately 500–1000 individual proteins in the fully assembled NPC [18]. The transport of salts, nucleotides, small molecules/proteins and components required for the syntheses of DNA and RNA occurs passively by diffusion through the NPC. In contrast, proteins larger than 40–65 kDa must be transported into the nucleus through the NPC with the assistance of transport receptors. These receptors recognize the transport signals that are present on cargo proteins allowing their import (reviewed in [19]). The key function of the NPC is to form a diffusion barrier between the cytoplasm and the nuclear compartment and to enable the nucleo-cytoplasmic traffic of macromolecules. In addition, the NPC is also involved in other nuclear processes that include DNA repair [20], the cell cycle [21], chromatin organization [22], transcription regulation [23,24], epigenetic memory [25] and RNA maturation and quality control [26].

Nucleoporin proteins

Despite its enormous dimensions, the NPC is built from a surprisingly small number of proteins called nucleoporins (Nups) [18]. The NPC displays a high degree of internal symmetry and can be divided into a symmetric part, enclosed in the nuclear membrane, and an asymmetric part, with extensions into the nucleus or cytoplasm (Fig. 1). Nups from the symmetric part of the NPC are generally classified into three categories: membrane-anchored (POMs, part of the nuclear envelope), scaffold (coat Nups and adaptor Nups) and channel (barrier Nups). Each category has unique structural features that are essential to execute specific functions. The Nups that form the asymmetric part of the NPC are called nuclear basket Nups and cytoplasmic filament Nups.

Membrane Nups

Membrane Nups (POMs) anchor the symmetric part of the NPC to the pore membrane where the inner and outer nuclear membranes fuse to form the nuclear pore binding the assembly complex to the NE. Membrane Nups contain transmembrane α-helices that allow the protein to anchor onto the membrane while large regions extend toward the luminal and pore sides of the membrane [27,28].

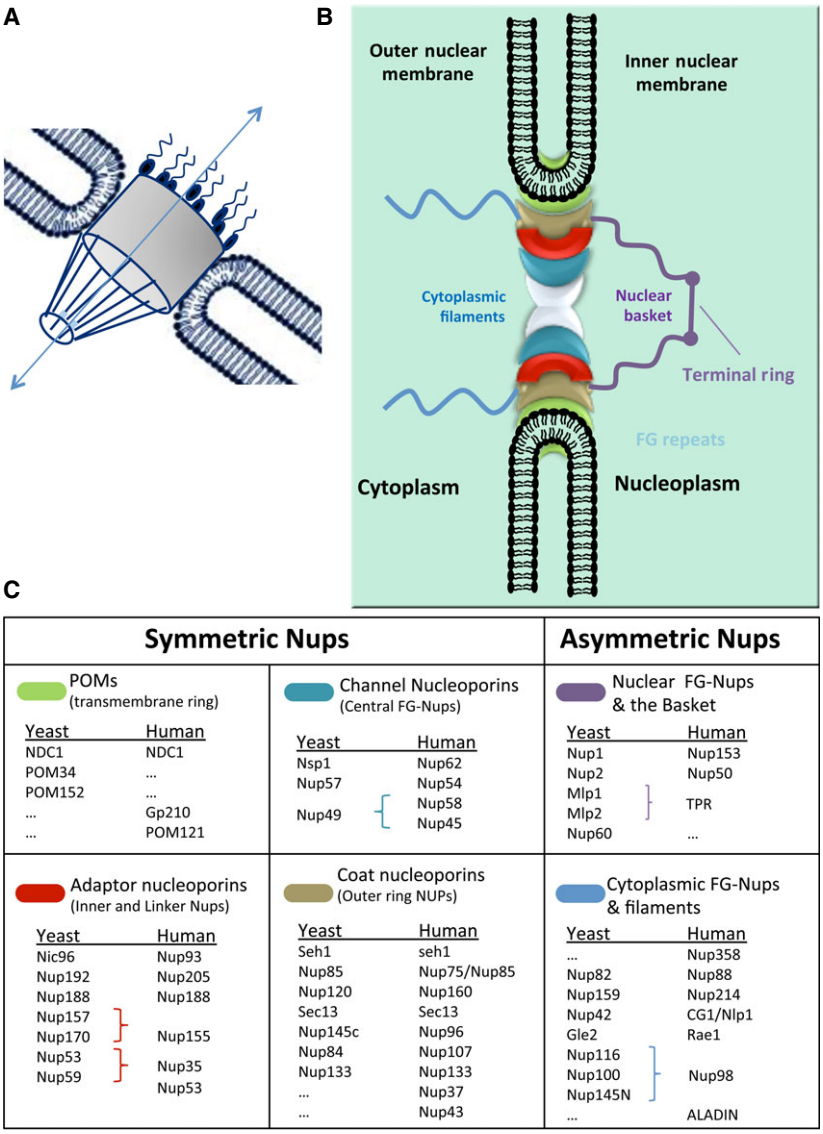

Fig. 1. Overall structure and molecular composition of the nuclear pore complexes (NPCs). (A) General structural features of the NPC. (B) A schematic model of the NPC. In this model, the NPC is divided into several groups according to their location and structural characteristics. The symmetrical core is composed of membrane-anchored POMS (transmembrane ring), channel Nups (central FG-Nups) and scaffold Nups composed by adaptor Nups (inner and linker Nups) and coat Nups (outer ring). Asymmetric parts of the pore are the nuclear FG-Nups and the basket plus the cytoplasmic FG-Nups and filaments. (C) The yeast and vertebrate homolog Nups that are known to constitute each NPC substructure are listed. Symmetric Nups are equally distributed on the cytoplasmic and nucleoplasmic parts of the NPC and form the core region. Asymmetric Nups form the nuclear basket and the cytoplasmic filaments. They serve as docking sites for transport factors and include associated mRNA export factors. See the main text for more information.

Scaffold Nups

The scaffold Nups (coat Nups and adaptor Nups) form the skeleton of the NPC connecting the membrane Nups to the barrier Nups. The scaffold Nups contain mainly α-solenoid and β-propeller folds and are classified into outer ring Nups, inner ring Nups and linker Nups according to their location and function. The high flexibility of the outer ring Nup components allows for conformational changes of the scaffold that enable large cargo to pass through the central channel [29].

Barrier Nups

Barrier Nups (channel Nups) are phenylalanineglycine Nups (FG-Nups) and form the innermost cylindrical layer that acts as a selective gatekeeper for nuclear transport regulation. FG-Nups contain multiple stretches of FG sequences that form intrinsically disordered regions. These motifs are present in about one-third of Nups forming an unstructured meshwork lining the central channel. The tentacle-like structures provide several low affinity, high specificity interactions with transport receptors that escort cargo proteins through the nuclear pore. Collectively, FG-rich regions build the diffusion barrier of the NPC.

Asymmetric Nups

Asymmetric Nups (formed by nuclear basket Nups and cytoplasmic filament Nups) are key components in establishing the directionality of the nucleo-cytoplas-

mic transport process. These structures mediate specific interactions with transport complexes and several asymmetric Nups that contain FG repeats serving as binding sites with important roles in cargo–NPC interactions.

The transport signals

For nuclear import and export, macromolecules generally require specific transport signals, namely a nuclear localization signal (NLS) or a nuclear export signal (NES) (Fig. 2). Soluble transport receptors of the karyopherin family of proteins (known as importins and exportins) recognize these sequences within macromolecules. NLS and NES can be defined as sequences within proteins that are necessary and sufficient for their import/export (summarized in Table 1). These sequences bind transport receptors either directly or via adaptor molecules and enable the release of the transport complex at the end of the translocation process [30]. The transport signals that interact with importin-α (Imp-α), importin-β (Imp-β), CRM1 (chromosome region maintenance 1, also known as exportin1 or Xpo1) and transportin-1 (also known as karyopherin-β2) are well described but they remain to be determined for the other 16 human karyopherin-βs [31–35]. The known NLSs can be classified into either classical NLSs or non-classical NLSs. The classical NLSs can be further divided into monopartite or bipartite NLSs. The non-classical NLSs include proline-tyrosine NLSs (PY-NLSs) that allow the cargo protein to bind to karyopherin-β2 that mediates the direct interaction with Imp-β. For nuclear export, the leucine-rich NES (recognized by CRM1) is the most extensively characterized export signal while the structure of the CRM1-snurportin1 and RanGTP complex has been elucidated [36,37].

Classical NLSs

The first nuclear transport signals were described in the SV40 large T antigen and nucleoplasmin in the early

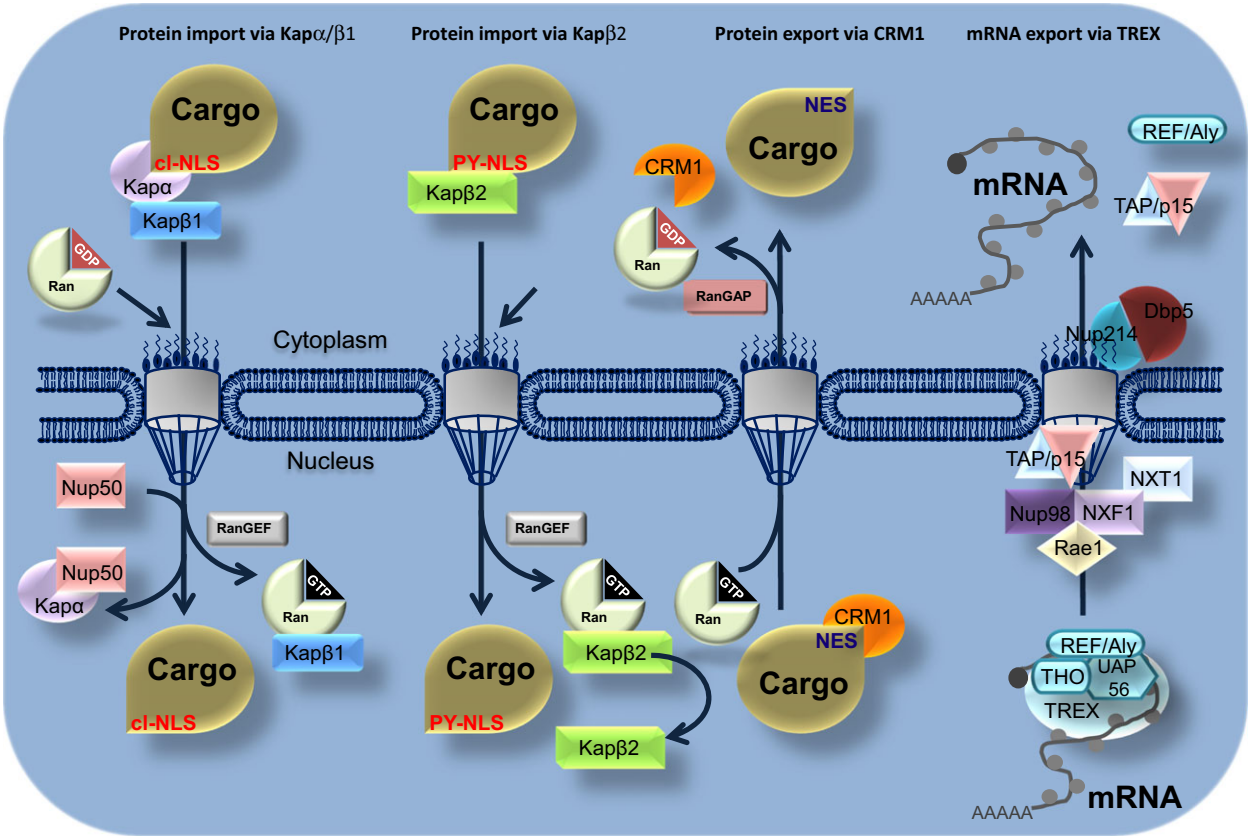

Fig. 2. Schematic overview of Ran-dependent nucleo-cytoplasmic transport. Nuclear export. CRM1 exports a great part of NES-containing protein. Nuclear import. Importin-α (Imp-α)/importin-β (Imp-β) heterodimer (designated as α and β) and karyopherin-β2 mediate the import of NLS-containing proteins. See the main text for details.

Table 1. Examples of different types of nuclear transport signals.

Amino acid sequence	Protein	Type of signal	References
PKKKRKV	SV40 T antigen	Classical NLS	[33]
KRx{10}KKKL	Nucleoplasmin	Bipartite NLS	[31,33]
VRILESWFAKNIENPYLDT	Matα2	Polar/nonpolar residues NLS	[196]
PAAKRVKLD	c-Myc	cMyc-NLS	[197]
YNDFGNYNNSSNFGPMKGG NFGGRSSGPYGGGGQY	hnRNP A1 (M9 sequence hydrophobic subclasses)	hPY-NLS	[198]
KVSRRG-GHQNSYKPY	hnRNP D (basic enriched)	bPY-NLS	[45]
RQARRNRRRRWR	VIH Rev protein	Arginine-rich NLS	[199]
DNSQRFTQRGGGAVGKNRRG GRGGNRGGRNNNSTRFNPLAK	Nab2p	Arginine/glycine-rich NLS	[200]
KTPGKKKKGK	Parathyroid hormone-related protein (PTHrP)	Lysine-rich-NLS	[201]
QDLNSTAAPHPRLSQYKS KYSSLEQSERRRRL	Snurportin1	UsnRNPs-NLS	[202]
LPPLERLTL	HIV Rev	Hydrophobic-NES	[32]
LALKLAGLKI	PKI	NES	[35,203]
LCQAFSDVIL	Cyclin B1	NES	
LQKKLEELEL	MAPKK	NES	[204]
LAEMLEDLHI	NMD3	NES	[77]

1980s comprising a short lysine-rich sequence classified as classical NLSs [31,38]. These bind the armadillo (ARM) domain in the C terminus of Imp-α. The adaptor protein Imp-α also binds the transport receptor Imp-β through its N-terminal αIBB domain forming a ternary complex [39]. The small size and relatively simple sequence patterns of these basic NLSs have facilitated identification of similar signals in many proteins. The classical NLSs contain one or two clusters of positively charged amino acids, typically lysine or arginine, and are divided into two classes, monopartite and bipartite classical NLSs [40]. The monopartite classical NLS is a short and highly basic signal. The two stretches of basic amino acids within a bipartite classical NLS are separated by a linker region that is usually 10–12 residues long, although longer linker sequences have been reported [41]. In addition, several atypical NLSs that bind to classical NLS binding sites in Imp-α have been characterized, including the hydrophobic NLS from phospholipid scramblase 1 [40].

Non-classical NLSs

Some cargo proteins bypass the requirement for an adaptor protein and bind directly to transport receptors through non-classical NLSs. Proteins that are directly recognized by Imp-β include ribosomal proteins, CREB, the human immunodeficiency virus (HIV) Rev and Tat, SREBP-2, the human T-cell leukemia virus type 1 (HTLV-1) protein Rex, PTHrP, cyclin B1, Smad3, TRF and SRY [40]. In contrast to the interaction between classical NLS and Imp-α, proteins that bind to Imp-β directly do not obey strict rules and the NLSs that confer Imp-β recognition vary significantly in both size and charge [40].

Many proteins that are imported into the nucleus can bind directly to the transport receptor transportin-1. Common characteristics between the apparently disparate signals recognized by transportin-1 were described unifying them into a new class of NLS termed PY-NLS [34]. The consensus motif of PY-NLS consists of a loose N-terminal hydrophobic motif, a central arginine residue and a C-terminal PY sequence [42]. The physical rules that describe the PY-NLSs as structurally disordered in free cargoes, positively charged and with weak consensus motifs predicted approximately 100 candidate transportin-1 cargoes [34]. Several of these have been confirmed experimentally [34,43,44]. Interestingly, arginine–glycine-rich NLSs known as RG-NLSs in the yeast proteins Hrp1 and Nab2 and the 38 amino acid long NLS of the hnRNP A1 protein, designated M9, were also shown to have the same characteristics as the PY-NLS [42,45]. Recently other non-classical NLSs such as the extensive coiled-coil domain of STAT5a [46] have been characterized. Furthermore, a number of proteins have been identified that contain both classical and non-classical NLS motifs that can interact directly with both Imp-α and Imp-β family members.

Leucine-rich NESs containing cargoes

The first signals that direct the nuclear export of a protein were identified in HIV Rev and protein kinase

inhibitor A [32,35]. The consensus sequence for NES is $\phi1\text{-}X_{(2-3)}\text{-}\phi2\text{-}X_{(2-3)}\text{-}\phi3\text{-}X\text{-}\phi4$ (where ϕ represents one of the hydrophobic residues L, V, I, F or M, and X can be any amino acid but preferentially is charged, polar or a small amino acid) [47–49].

More complex export signatures incorporate the three-dimensional features of the whole protein that are recognized and targeted for export [50,51]. Proteins containing these NESs are recognized and exported by CRM1 [52–55] (Fig. 2). Güttler *et al*. showed that CRM1 contains five pockets for binding the conserved hydrophobic residues of NESs. Accordingly, a structure-based NES consensus with an additional hydrophobic position (five instead of four) has been proposed [56]. Kosugi *et al*. generated a large number of NES peptides in a random peptide library screen and delineated multiple distinct consensus sequences that described more than 80% of known functional NESs [57]. A predictor, NESsential, that uses sequence derived meta-features, such as predicted disorder and solvent accessibility, in addition to a primary sequence that can identify promising NES-containing candidate proteins [58,59], http://validness.ym.edu.tw/index.php. Other recently developed NES predictor tools include WREGEX, which uses position-specific scoring matrices for motif prediction [60], and NESMAPPER (http://sourceforge.net/projects/nesmapper), which has been developed based on the activity profile of all classes of NESs [61].

Nuclear transport receptors

Proteins larger than 40 kDa are transported through the NPC by soluble nuclear transport receptors (NTRs). NTRs continuously shuttle between the nucleus and the cytoplasm, bind their cargo on one side of the nucleus and release it on the other side. The majority of the nuclear-cytoplasmic transport receptors are members of the β-karyopherin protein family with each member recognizing a unique group of cargo proteins or RNAs (summarized in Table 2) [62]. There are 22 putative members of the karyopherin family in humans [63] that share only modest sequence homology with the greatest homology noted within their Ran-binding domain [64]. A specific architecture within the karyopherin family is the tandem HEAT repeat fold formed by anti-parallel helices that are linked by a short intra-repeat loop [65]. These repetitive structures are inherently flexible and contribute substantially to Ran-controlled cargo recognition and cargo release [66]. Karyopherin-β proteins can either directly or indirectly, through adaptors, interact with their cargo. In addition there are six homologs of adaptor proteins that belong to the Imp-α protein family that have been identified in humans which adds a further level of complexity to this process [67]. For example, Imp-β mediated nuclear import of the uridine-rich small nuclear RNPs involves a different adaptor protein called snuportin-1 and, as in the case of Imp-α, snuportin-1 binds Imp-β through an IBB domain. The karyopherin-β proteins are multi-domain transport factors that contain a cargo binding domain, an NPC binding domain [68] and a binding domain for the small Ras-like GTPase Ran [69,70]. Family members contain both import and export receptors; however, only a few of them have been functionally characterized in higher eukaryotes (indicated in Table 2).

Import receptors

A protein that contains a classical NLS is bound by the NLS binding pocket formed by the armadillo repeats within the Imp-α adaptor protein. The transport receptor Imp-β binds to Imp-α and then targets the NLS-containing protein into and through the NPC. Intriguingly, Imp-β has been shown to contain two binding sites for FG-rich motifs that are located away from the cargo binding site [71]. Most karyopherin-β proteins do not rely on adaptors to bind to cargoes as they can directly interact with basic NLSs of core histones and ribosomal proteins or arginine–glycine-rich NLSs of some RNA binding proteins.

Export receptors

CRM1 (chromosome region maintenance 1)

The most extensively characterized export receptor is chromosome region maintenance 1 (CRM1), a member of the karyopherin-β family of receptor proteins. CRM1 exports proteins that contain leucine-rich NESs from the nucleus into the cytoplasm [53,55]. CRM1 has 20 HEAT domains that allow RanGTP to bind. Cargo binding takes place outside of this HEAT domain ring in a hydrophobic cleft producing a generic NES docking site [56]. CRM1 recognizes NESs that are present in a large range of proteins that are structurally unrelated [72]. CRM1 can also be recruited by adaptor molecules in situations where it does not bind directly to a protein that is to be exported from the nucleus (e.g. Exp5 cooperates with CRM1 to export large ribosomal subunits [73]). CRM1 also participates in the export of the 40s and 60s pre-ribosomal subunits as well as essential RNPs [74–77]. With two adaptors [the Cap binding complex

Table 2. Transport receptors and cargoes.

Vertebrate karyopherins	*Saccharomyces cerevisiae* karyopherins	Cargoes (selection) vertebrate (V) yeast (Y)
Imp-β/Kapβ1	Kap95	Classical NLS via Imp-α; cyclin B1; snurportin1; SRY; PTHrP; CREB, AP-1, TRF1, Smad3; SREBP-2; HTLV-1 Rex, HIV-1 Tat, HIV-1 Rev; NF-YA; adenovirus core protein pVII; aristaless/arx; cJun; H1; HPV16E6; H1; H2A; H2B; H3; H4; rPL23a, rPS7, rPL5, rPL18a, rPL6, rPL4; PP2A (PR65)
Kapβ2 (Transportin/ Transportin-2)	Kap104	(V) PY-NLS cargoes; PQBP-1, YBP1, PABP2, EWS, FUS, SAM68, hnRNP M, hnRNP A1, hnRNP A0, hnRNP A2, hnRNP A3, hnRNP D, hnRNP F, JKTP-1, TAP (NXF1), HuR, HEXIM1, RB15B, Clk3, WBS16, Cyclin T1, TAFII68, CPSF6, HCC1, ETLE; tfg2p non PY-NLS cargoes: TAFI48; NPM-ALK, SRP19; H2A; H2B; H3; H4; c-Jun; rPL23a, rPS7, rPL5; adenovirus core protein pVII; HIV-1 Rev; HPV16 E6, HPV16 L2, HPV18 L2 (Y) Nab2p; Hrp1p; Tfg2p
Importin-5 (Kapβ3 or RanBP5)	Kap121 (Pse1)	(V) p60TRP;Rag-2; PGC7/Stella; apolipoprotein A-I; influenza A PB1-PA; HPV18 L2; HPV16 L2; CDK5 activator p35; TAFI48, c-Jun; HIV-1 Rev; rPL23a, rPS7, rPL5, rPS3a; H2A; H2B; H3; H4 (Y) Aft1p; Asr1p; Egd1p; Nop1p, Nup53p; Pdr1p; Pho4p; Sas2p; Sof1p; Spo12p; Ste12p; Yap1p; Yra1p; secondary pathway for histones, ribosomal proteins, Ho, SRP, TBP
Importin-4 (RanBP4)	Kap123	(V) Vitamin D receptor, TP2, HIF1-α, rPS3a (Y) Egd1p; H3 (Hht2p, H4 (Hhf2p); Sas2p; SRP; Rpl25p, Rp10a, Rps1p, Rpl4p, Rpl15p, Rpl16p, Rpl18p,Rpl25p, Rpl41p; secondary pathway for Asr1p, Asf1p, H2A, H2B, Htz1p, Yap1p, Yra1p, TBP
Importin-9	Kap114	(V) Hepatocellular carcinoma associated protein, HSP27, rPS3, rPS9, rPL19, rPL18a, rPS7, rPL6, rPL4; c-Jun; H2A, H2B, H3, H4; aristaless (Arx); PP2A (PR65); (Y) H2A (Hta1p), H2B (Htb1p, Nap1p, TBP (Spt15p); TFIIB Sua7p); rfp1; secondary pathway for Asr1p
Importin-7	Kap119 (Nmd5)	(V) Proline-rich homeodomain; EZI; RK-2, MEK1, Smad3; HIV-1 integrase; CDK5 activator p35; HIF1-α; c-Jun; glucocorticoid receptor;HIV-1 Rev; rPL23a; rPS7; rPL5; H2A, H2B, H3, H4; Imp-β/-7 heterodimer: H1; HIV-1 integrase, adenovirus core protein pVII; rPL6, rPL4, rPS3a; (Y) Crz1p; Gal4p; Hog1p; Ssa4p; TFIIS (Dst1p), Rpf1p; secondary pathway for histones H3 and H4
Importin-8 (RanBP8)	Kap108 (Sxm1)	(V) Ago2; Smad4, Smad1; NPM-ALK; SRP19; (Y) Lhp1p, Pab1p, Rpl16p, Rpl25p, Rpl34p; secondary pathway for Ho, histones H3 and H4
Transportin-SR (-SR2/-3/TNPO3)	Kap111 (Mtr10)	(V) ASF/SF2, SC35, TRA2α, TRA2β; HPV E2, RBM4, ALEX3, BAB71287, BAP1, MLF2, ODF2; dASF, dSC35, d9G8, Rbp1, B52, RSF1; HIV1 IN; (Y) Gbp2p; Hrb1p; Npl3p; tRNAs
–	Kap122 (Pdr6p)	(Y) Imports sc-cargo – Toa1 and Toa2, TFIIA
Importin-13	–	NF-YB/NF-YC; NC2α/NC2β; Myopodin, hUBC9, eIF1A Y14-Mago; glucocorticoid receptor CHRAC-15/CHRAC-17, p12/CHRAC-17; PAX6, Pax3, Crx; Aristaless (Arx); rPL5; histone fold heterodimers
CRM1 (Exportin-1)	CRM1 (Xpo1/Kap124p)	Leu-rich NES cargoes; HIV genomic RNA; m7G- capped UsnRNAs; 40S and 60S pre-ribosomal subunits via NMD3 adaptor; snurportin1 (SPN1)
CAS (Exportin-2)	Kap109 (Cse1)	Imp-αs (Y) Kap60/Srp1
Exportin-4/ Bidirectional NTRs	–	Sox-2, SRY; eIF5A, Smad3
Exportin-5	Kap142 (Msn5)	tRNA, eEF1A (via aa-tRNA); dsRNA-binding proteins (via dsRNA); pre-miRNAs; 60S pre-ribosomal subunits
Exportin-6	–	Actin-profilin complexes
Exportin-7 (RanBP16)	–	p50-RhoGAP, 14-3-3-σ
Exportin-t (Xpo-t)	Kap127 (Los1)	tRNA

(CBC) complex and PHAX] CRM1 is also essential for the maturation of the spliceosomal U snRNPs [78,79]. Furthermore, CRM1 actively maintains the exclusive cytoplasmic localization of RanBP1, RanGAP and other translation factors that contribute to the identity of the nuclear compartment [80–83]. Another key function of CRM1 is its role in SPN1 recycling (an adaptor for U snRNPs) back to the cytoplasm [51]. Intriguingly, overexpression of CRM1 has been reported in various tumor types and has been

correlated with poor prognosis and resistance to therapy [4,5]. In addition CRM1 is hijacked during infection by many viruses [32,84–86].

Other exportins

In addition to CRM1, a number of other alternative export receptors have been characterized. Exportin 2 recycles Imp-α from the nucleus into the cytoplasm allowing Imp-α to mediate another round of nuclear import if required [87–89]. Together with the adaptor STRADα exportin 7 regulates the distribution of LKB1 kinase [90] and regulates the leakage of RhoGAP1 and 14-3-3-σ into the nucleus [91]. Exportins are also dedicated to RNA transit. For example, exportin-t is dedicated to export fully mature tRNA with the 5′- and 3′-ends correctly processed [92–95]. A second tRNA (alone or with eEF1A) exporter is exportin 5 which displays a different binding specificity compared to exportin-t [80,96]. Exportin 5 also exports double strand RNA, pre-miRNAs [97–99] and cooperates with CRM1 as described previously. Exportin 4 exports eIF5A and Smad3 but can also act as an importin for Sox-type transcription factors [100,101]. Importin 13, despite its namesake, also demonstrates a bi-directional transit capability directing eIF1A nuclear export and the import of the heterodimer component of the exon junction complex Mago-Y14 [102,103].

Alternative nuclear transport pathways

While most proteins are transported through the NPC by conventional karyopherin-β mediated mechanisms, alternative nuclear transport pathways have been described for a number of proteins. These karyopherin-β-independent pathways include transport by alternative carriers such as the calcium-binding proteins calmodulin and calreticulin and translocation that seems to be mediated by direct interaction with NPC components, independent of carrier molecules. Importantly, many proteins are transported by more than one mechanism. It has been suggested that these seemingly redundant pathways may ensure the maintenance of cellular functions under conditions in which one pathway is inhibited [104]. Calmodulin has been shown to facilitate the nuclear import of the transcription factors SRY and SOX9 by binding to specific sequences in a way similar to NLS binding by karyopherin-β [105]. The nuclear import of these transcription factors is regulated by calcium. Similarly, calreticulin exports the glucocorticoid receptor from the nucleus in a calcium-dependent manner [106]. In addition, calreticulin is involved in the nuclear export of thyroid hormone receptor α1 and viral proteins [107–109].

Some proteins can enter the nucleus without requiring receptor proteins and there is growing evidence that receptor-independent nuclear import can be mediated by the direct binding of the transport cargo to FG-containing Nups. It has been suggested that proteins with armadillo repeats such as β-catenin, that are structurally related to karyopherin transport receptors, can directly bind to the NPC and are imported into the nucleus independently of conventional NTRs of the karyopherin-β type. Other proteins such as the tumor suppressor proteins SMAD3 and 4 and the transcription factor PU.1 utilize direct binding to FG-Nups to cross the NPC. Conversely, some proteins can be transported through their interaction with proteins that contain a functional NLS known as a 'piggyback' mechanism. Recently, Speese *et al.* reported a novel NPC-independent mechanism for the nuclear export of RNP by nuclear envelope budding akin to nuclear egress of herpes-type viruses [110]. During this mechanism, RNP granules bud into the perinuclear space in a manner dependent on lamin C.

The Ran system

The nucleo-cytoplasmic transport process mediated by members of the karyopherin protein family requires metabolic energy. The loading and unloading of transport receptors with cargo molecules is controlled by the small Ras-like GTPase Ran and requires GTP hydrolysis [111]. Ran is a 25 kDa protein that exists in two different nucleotide-bound states: RanGDP and RanGTP [112,113]. Ran hydrolyzes GTP very slowly and interacts with regulatory proteins including RanGAP1 (Ran GTPase activating protein 1) and RanBP1 that significantly increase GTP hydrolysis by Ran [114]. Conversely, the Ran regulatory protein RanGTP exchange factor (RanGEF, also termed RCC1 in human cells) accelerates the exchange of nucleotides restoring the pool of the RanGTP. RanGAP1 is exclusively cytoplasmic [115] and RanBP1 is predominantly localized in the cytoplasm [116] whereas RanGEF is nuclear. As a consequence of this strict nuclear localization of RanGEF and cytoplasmic RanGAP1, Ran in complex with GTP is localized mostly in the nucleus and RanGDP (Ran in complex with GDP) in the cytoplasm [62]. This RanGTP gradient provides directionality to nuclear-cytoplasmic transport because importins and exportins differ in the way they utilize the RanGTP gradient [117]. Import complexes are dissociated by RanGTP binding in contrast to export complexes which are formed by association with

RanGTP. Both importins and exportins bind RanGTP directly [52,118,119] and use the metabolic energy supplied by the RanGTPase system for directional transport [120,121]. Importins bind their cargo at low RanGTP level (in the cytoplasm) and traverse the NPC as dimeric complexes transporting cargo (Fig. 2) [118,119]. Exportins act in an opposite manner, recruiting their cargo at high RanGTP levels in the nucleus (Fig. 2) [84,88]. The RanGTP-exportin-cargo complex crosses the NPC into the cytoplasm where it disassembles following GTPase activation releasing the transported cargo. The free export in translocates back into the nucleus to mediate another round of export.

Trafficking of macromolecules through the NPC

A number of models have been proposed to describe the molecular mechanism of selective gating through the NPC. The polymer brush model suggests that movements of the unfolded FG-Nups sweep away macromolecules [11,122,123]. Conversely, the collapse model, based on atomic force microscopy data [124,125], suggests that regions of FG repeats may collapse following the binding of transport factors. These transport factors would open up their own passage through the central tube when they pass the meshwork of FG repeats. The 'hydrophobic gel' model [126], also called 'saturated model' [127,128], proposed that the phenylalanines in the FG repeat regions are crosslinked with each other and form a dense gel of FG repeat filaments. Transport factors bind to these FG repeats, dissolve the crosslinks and facilitate passage through the nuclear pore. Another model suggested that the FG repeat regions form a layer coating the inner walls of the central tube where non-binding molecules can only pass through the narrow FG-Nup-free middle and where the transport factors enter this layer through binding giving them full access to the tube volume [129]. Melcák *et al.* proposed a model of pore dilatation by intermolecular sliding of Nup58/45 tetramers to adjust the diameter of the transport channel for the passage of cargo [130].

Nuclear import

The recognition of NLSs by Imp-α and heterodimerization with Imp-β initiates the process of nuclear import. The import complex, consisting of the cargo protein and the Imp-α/β complex, localizes to the nuclear envelope, binds RanGDP and docks at the nuclear pore. The Imp-α/β complex mediates the binding to and translocation through the NPC. Once translocation through the pore is executed, dissociation of the import complex is stimulated by RanGTP in the nucleus. Importin-α is recycled back to the cytoplasm through the nuclear exporter CAS, whereas Imp-β is separately transported back to the cytoplasm together with RanGTP. RanGAP1-facilitated GTP hydrolysis of Ran on the cytoplasmic side causes the release of Imp-β for the next cycle.

Nuclear export

The process of nuclear export is carried out according to principles which are analogous to those of nuclear import, using specific nuclear export receptors like CRM1 that recognize NES sequences on cargo proteins [131,132]. The affinity of CRM1 for most NESs is low and formation of the export complex is promoted by RanBP3 which links CRM1 to the chromatin binding protein RCC1 [133,134] and increases the active concentration of RanGTP. This promotes the affinity of the NES cargo for the export receptor [135,136]. This complex moves through the NPC via the interaction with FG repeat proteins [137]. Within the cytoplasm, the CRM1 complex binds to the cytoplasmic filament complex (Nup88, Nup214 and Nup358 [84,138–141]) and interacts with RanGAP causing the hydrolysis of GTP which promotes the dissociation of the protein complex. Following this dissociation, the cargo is released in the cytoplasm [36,142].

Export of RNAs

RNAs transcribed in the nucleus have to be exported, either to fulfill their function in protein synthesis or to mature into functional particles [143]. The pre-mRNA is processed and packaged into messenger ribonucleoprotein (mRNP) complexes to be exported through bulk or specific export. The majority of poly-A transcripts are exported via the non-karyopherin heterodimer Nxf1/Nxt1 independently of the RanGTP gradient. Conversely, a specific subset of endogenous transcripts is exported from the nucleus via CRM1. For efficient export, RNAs must undergo processing that includes splicing, 3′-end formation of the poly-A tail and the addition of a methyl-7-guanosine (m7G) cap structure to their 5′-ends [144–146]. For the majority of transcripts, the m7G cap recruits the CBC, which then activates export factors that allow the export mRNPs to bind to the NPC and traverse the hydrophobic central channel. Nxf1 (also known as TAP) [147]) is the major driver of interaction between the export mRNP and the NPC. The Nxf1/Nxt1 hete-

rodimer is recruited to the mRNP via the transcription-export (TREX) complex [79,144,145, 148–150]. The TREX complex consists of UAP56, REF/a Aly, CIP29 and THO multi-subunit complex composed of THOC1/Hpr1, hTho2, THOC5, THOC6, THOC7 and Tex1 [144,151–153]. REF/Aly and THO complexes promote the interaction of cargo mRNAs with the Nxf1 receptor [144,151] that associates mRNPs with the nuclear basket via the ribonucleic acid export protein Rae1 and Nup98 to permit passage through the central channel (Fig. 2) [154,155]. At the cytoplasmic face, the cargo mRNPs are released and the export factors are recycled. RanBP2 associates with Nup88 and Nup214 [141] and plays a very important role in cargo release and recycling after bulk mRNA export.

An alternative to the Nxf1-driven export pathway involves the serine- and arginine-rich SR proteins SRp20 and 9G8 that mediate the export of H2a mRNA and of some spliced transcripts [156,157]. Although the majority of mRNAs use the Nxf1 receptor to cross the NPC, subsets of transcripts are exported via the general CRM1 pathway. Additionally, through protein cofactors, CRM1 is involved in the export of specific types of mRNAs, small nuclear RNAs (U snRNAs) and ribosomal RNAs. CRM1 does not bind RNA directly but via NES-containing adaptor proteins that bind to RNA or other RNA binding proteins [148]. Once this step is completed, CRM1–cargo complexes dissociate, permitting the RNA to enter the cytoplasm and to recycle export factors [148]. As in bulk mRNA export, Nup88, Nup214 and RanBP2 play critical roles in the recycling and release steps for CRM1-dependent export [138,158].

Regulation of the nucleo-cytoplasmic trafficking

The nucleo-cytoplasmic transport processes are regulated by cellular signaling systems via cargo protein modification(s) and the transport machinery, including the transport receptors, the NPC and the Ran system. Indeed targeting nucleo-cytoplasmic transport has directed the development of new exciting therapeutic avenues to treat cancers that display aberrant protein subcellular localization [159,160].

Modification of the cargo proteins

Importin-NLS/exportin-NES interactions can be modulated by conformational changes in both NLS/NES regions and in the substrate binding site of karyopherins [161]. In some cases the three-dimensional structure of a protein masks its own transport signal as in the case of p105 [161], the precursor p50 subunit of NF-κB. Upon stimulation, p105 is phosphorylated, the C-terminal part of the protein is degraded and the NLS becomes accessible allowing the p50 protein to be recognized by the Imp-α/Imp-β complex [162]. Furthermore, phosphorylation within or close to the NLS/NES can promote intramolecular masking. In the case of Hog1p (high osmolarity glycerol pathway-signaling protein) phosphorylation at Thr174 and Tyr176 renders Hog1p-NES inaccessible for binding to CRM1 and prevents its export from the nucleus [163]. Similarly NF-AT2 (nuclear factor of activated T cell 2) contains two NLSs that can be masked by phosphorylation at a low calcium concentration. Following a calcium concentration increase, calcineurin is dephosphorylated and NF-AT2 is imported into the nucleus [164–166]. In the canonical NF-κB pathway, I-κB masks the NLS sequence of NF-κB p65 [167]. In resting conditions, I-κB is phosphorylated, ubiquitinated and degraded by proteasome resulting in NLS unmasking and NF-κB p65 nuclear import [168,169]. One of the multiple regulatory mechanisms of p53 is its homo-tetramerization that masks one of its NES sequences. Dissociation of this tetramer is required for its nuclear export [170]. Binding of proteins to RNA and DNA can also modulate the intermolecular masking of localization signal. For example, the NLS of the Rev protein, implicated in HIV mRNA translocation, is masked when Rev is linked to mRNA and recycling of Rev is possible only after release of the transported mRNA [171]. Conversely, nucleo-cytoplasmic transport can be enhanced by phosphorylation of SV40 virus large T antigen [172] or Pho4 factor [173] increasing the affinity of their NLS/NES signals to the corresponding karyopherin receptors [172].

Viral regulation of nucleo-cytoplasmic trafficking

Many viruses have evolved elegant strategies to exploit the host nucleo-cytoplasmic transport pathways to evade the cellular anti-viral response or to facilitate viral replication. Some viruses such as the vesicular stomatitis virus interact with Nups to inhibit the export of host mRNAs that encode anti-viral factors and make the translation machinery available for expression of viral mRNAs [174]. Similarly, the ICP27 protein of herpes simplex virus interacts with Nup62 and blocks nuclear import of proteins via Imp-α/β1 and Imp-β2 pathways [175,176]. Conversely, poliovirus and human rhinovirus block the nuclear import of proteins via the Imp-α/β1 and Imp-β2

pathways by viral-mediated proteolytic cleavage of specific Nups [21,177–183]. Other viruses inhibit the nuclear import of the STAT proteins that are known to be key regulators of the cell anti-virus response. Furthermore, the severe acute respiratory syndrome (SARS) virus disrupts the nuclear import of STAT1 by tethering the tyrosine-phosphorylated STAT1–Imp-α/Imp-β complex to endoplasmic reticulum/Golgi membranes [184–187]. Yet another way exploited by viruses to prevent STAT1 nuclear localization is to bind viral proteins to import receptors. The Ebola virus VP24 protein binds Imp-α to block its interaction with phosphorylated STAT1 and hnRNP C1/C2 [188–190]. The L1 protein of the human papilloma virus type 11 binds Imp-β2/β3 and disrupts cargo import. Encephalomyocarditis virus exerts its inhibitory effect on the nuclear protein import of infected cells via its L protein that hyper-phosphorylates Nups and binds Ran [191–193]. In addition herpes viruses and HIV promote viral mRNA export by reprogramming the cellular transport pathways. The HIV-1 Rev protein facilitates nuclear export of unspliced or partially spliced viral mRNAs through the Rev-responsive element, an RNA signature within these viral mRNAs [85,194,195]. As a result, Rev-bound viral RNA binds to CRM1 and RanGTP and is transported through the NPC.

Conclusions

The nuclear transport processes within our cells are governed by several types of protein–protein interactions (e.g. adaptor protein–cargo, adaptor protein–transport receptor, transport receptor–cargo, transport receptor–Ran, transport receptor–Nup), yet only one enzymatic reaction occurs, namely the hydrolysis of GTP by Ran GTPase. While pharmacological targeting of protein–protein interactions has historically been considered challenging, the development of nuclear export inhibitors proves the viability of this approach. Therapeutic agents that attempt to normalize or to target protein localization are aimed at various regulatory components within the transport process, including the upstream regulatory components, the cargo proteins, the transport receptors, the Ran regulators and the NPC itself. In some cases, compounds have been developed to successfully influence subcellular protein distribution in disease states, including CRM1 and Imp-α/β inhibitors. Continued and intensified research efforts aimed at better understanding the nuclear transport mechanisms, and how they relate to pathogenesis, will probably reveal the identity of novel targets for the treatment of cancer and to subvert viral infections.

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