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Review

Nucleocytoplasmic transport: taking an inventory

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Abstract. In eukaryotic cells, the enclosure of the genetic information in the nucleus allows the spatial and temporal separation of DNA replication and transcription from cytoplasmic protein synthesis. This compartmentalization not only permits a high level of regulation of these processes but at the same time necessitates a system of selective macromolecular transport between the nucleus and the cytoplasm. Transfer of macromolecules between both compartments is mediated by soluble receptors that interact with components of nuclear pore complexes (NPCs) to move their specific cargos. Transport occurs by way of a great variety of different pathways defined by individual receptors and accessory factors. Often, processes in substrate biogenesis that precede transport concurrently recruit transport factors to substrates, thus making transport responsive to correct and orderly synthesis of substrates. Some current challenges are to understand how transport factor-substrate interactions are controlled and integrated with sequential steps in substrate biogenesis, how large macromolecular complexes are restructured to fit through the NPC channel and to understand how transport factor-NPC interactions lead to actual translocation through the NPC.

Key words. Nucleocytoplasmic transport; import; export; nuclear pore complex; mRNA; ribosome.

Fundamental aspects

Introduction

The exchange of material between the nucleus and cytoplasm occurs by way of nuclear pore complexes (NPCs). The NPC is a massive structure ~15–30 times the size of a eukaryotic ribosome [1, 2]. Completely spanning the two membranes that separate the nucleus from the cytoplasm, the NPC houses a central ~65-nm-long aqueous channel. The NPC appears to remain open to free diffusion of low molecular weight solutes, bringing the nucleus and cytoplasm into continuous ionic contact. In contrast, proteins and RNA molecules generally do not diffuse across the NPC. Rather, like a parcel delivery service, macromolecules are carried through the central channel of the NPC by way of specific transport receptors (refer to fig. 1). These transport receptors identify their substrates by recognizing specific signals present within the cargo molecules. The shepherding of cargos by trans-acting carriers clearly is a means to allow accumulation of materials against their chemical potentials. Not surprisingly then, transport requires input of energy, which is usually derived from GTP hydrolysis; however, the expenditure of high-energy phosphate is not used directly to move receptor-cargo complexes through the NPC. Rather, during transport, GTP is consumed by Ran (Gsp1p in yeast), a member of the Ras-related GTPase superfamily. The interaction between RanGTP and nuclear transport receptors dictates in which compartment a given receptor will bind or release its cargo. RanGTP-re-

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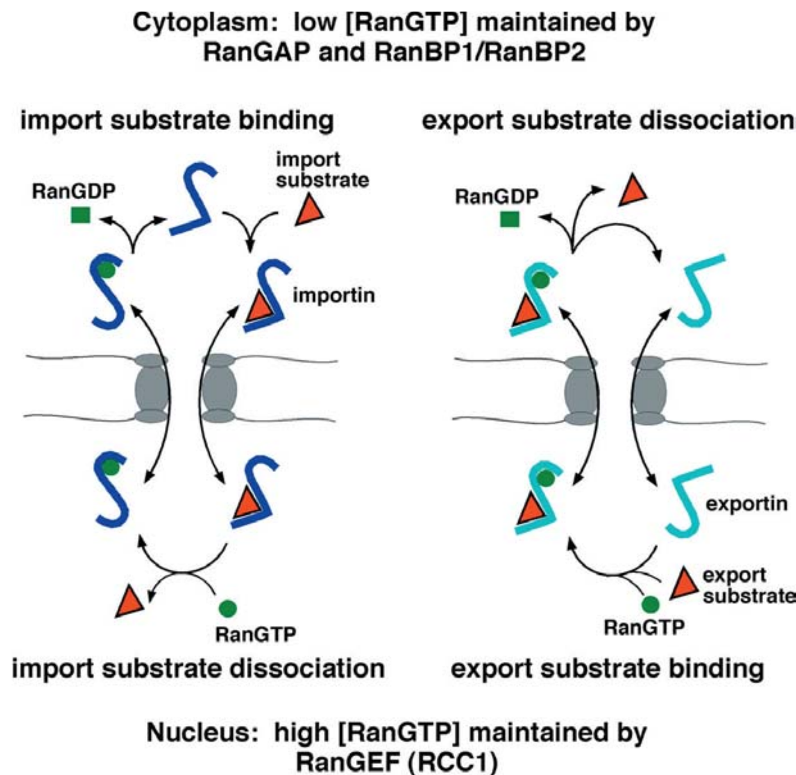

Figure 1. Control of cargo binding to importins and exportins by Ran. Transport receptors mediate nuclear pore passage of substrates by a diffusional mechanism involving contacts with FxFG-containing nucleoporins. The directionality of the transport reaction is dictated by the differential localization of RanGTP. In the nucleus, the RanGTP concentration is kept high by chromatin-associated RanGEF. In the cytoplasm, RanGAP, aided by RanBP1 and/or RanBP2, converts RanGTP to RanGDP. In general, importins (dark blue) have a high affinity for GTP-bound Ran. RanGTP binding to importins causes dissociation of the import substrate. Importins bind a substrate (triangle) in the cytoplasm where the RanGTP concentration is low. Upon arrival in the nucleus, they associate with RanGTP, and most likely a conformational change in the substrate-binding domain leads to cargo release. The importin/RanGTP complex translocates back to the cytoplasm where RanGAP/RanBP1/RanBP2 dissociate RanGTP from the importin. Most exportins (light blue) have a low affinity for RanGTP and substrate. Both bind cooperatively to the exportin in the nucleus. Conformational changes in the transport receptor lead to the formation of a stable export complex. Export complex formation in the nucleus is favored by the high nuclear RanGTP concentration. After export to the cytoplasm, the export complex is disassembled by RanGAP assisted by RanBP1 and/or RanBP2. The exportin reenters the nucleus on its own.

sponsive transport accounts for the majority of nucleocytoplasmic exchange of macromolecules; however, Ran-independent transport receptors also exist.

A still useful paradigm to illustrate the general principles of nuclear transport and the way in which RanGTP and GTP hydrolysis on Ran confer directionality is the first transport pathway that was delineated, involving the import receptor importin β, which mediates the import of proteins containing short, basic nuclear localization signals (NLSs) [3].

Unlike many other transport signals (see later), the short, basic NLSs do not bind importin β directly. Instead, these NLSs are recognized by a transport adaptor, importin α, that associates with importin β by virtue of its N-terminal importin β binding domain (IBB) [4–6]. The trimeric complex consisting of the NLS-protein/importin α/importin β is targeted to the NPC by importin β [7–9]. This receptor-cargo complex then translocates to the nuclear side of the NPC by interaction of importin β with NPC constituents. In the nucleus, importin β binds RanGTP, which causes release of the importin α/cargo protein complex [10–12]. The cargo protein itself is released from importin α since, in the absence of importin β binding, the affinity of importin α for NLS cargo drops due to auto-inhibition by the IBB domain, which folds back into the NLS substrate binding pocket of importin α [13]. Importin β, still bound to RanGTP, is recycled back to the cytoplasm, again by translocation through the NPC central channel. Importin α cannot translocate through the NPC on its own; thus, importin α binds to a specific export receptor, CAS [14]. The affinity of CAS for importin α is dramatically enhanced by cooperative binding with RanGTP. The CAS/RanGTP/importin α complex then travels through the central channel to the cytoplasm. In the cytoplasm, importin β- and CAS-bound Ran is stimulated to hydrolyze its GTP, causing dissociation of Ran from the export complexes. Dissociation of Ran from importin β permits importin β to form a new trimeric import

complex with cargo and importin α, while dissociation of Ran from CAS releases importin α, permitting CAS to return on its own to the nucleus.

Importin β and CAS are members of the *importin β/karyopherin* superfamily of nuclear transport receptors. The importin β/karyopherin family mediates the majority of nuclear transport processes in metazoans and yeast. All members of this superfamily perform three functions: (i) they bind cargo by way of their nonconserved C-terminal domains, (ii) they use RanGTP binding through their weakly conserved N-terminal domains to control cargo association and (iii) they bind a specific group of proteins, the FG-repeat nucleoporins, contained within the NPC. These aspects of importin β/karyopherin receptors will be discussed in detail below. Also, as noted above, while RanGTP-responsive transport accounts for the majority of nucleocytoplasmic exchange of macromolecules, other Ran-independent transport receptors exist, and their properties will be examined as well.

In summary, for the most part, factors that transfer cargo from the cytoplasm to the nucleus, which are referred to as importins, require an environment free of RanGTP to bind their cargo, a condition satisfied in the cytoplasm, and they release their cargo in the nucleus in which RanGTP is plentiful; conversely, factors that transfer cargo from the nucleus to the cytoplasm, exportins, require RanGTP to bind their cargo, and they release their cargo in an environment in which RanGTP is converted to RanGDP. Thus, while importins and exportins likely navigate the central channel by the same mechanism, it is the asymmetrical distribution of RanGTP and the opposite responses of transport receptors to RanGTP in cargo binding that regulate the direction of cargo movement and allows for receptor recycling.

In the next section we begin by describing the various factors responsible for producing and compartmentalizing RanGTP. We will then address still unresolved questions pertaining to the mechanisms of nucleocytoplasmic transport, in particular, how the structure of the NPC mediates transport. This will be followed by a discussion of how transport receptors identify their cargo and respond to RanGTP. Finally, we examine the specific pathways responsible for transport of different classes of macromolecules, with emphasis on transport pathways that are just beginning to be understood.

The RanGTPase system

As indicated, crucial for the directionality of nuclear transport is the asymmetrical distribution of RanGTP between the nucleus and cytoplasm. The maintenance of this asymmetry involves the spatial separation of regulators that control Ran's nucleotide state and its subcellular location.

RanGAP

Ran hydrolyzes GTP very slowly; the half-life of the RanGTP complex is several hours [15, 16]. This feature of Ran is but one means to ensure that RanGTP concentrated in the nucleus remains as RanGTP. In the cytoplasm, where GTP hydrolysis must occur, the Ran-specific GTPase activating protein, RanGAP1 (Rna1p in yeast), increases Ran's rate of GTP hydrolysis by five orders of magnitude [15, 17]. As required for the asymmetrical distribution of RanGTP, RanGAP is confined to the cytoplasm. Yeast Rna1p contains a nuclear export signal that binds to the export factor Xpo1p, which rapidly exports any Rna1p that might enter the nucleus [18]. Mammalian RanGAP's localization is restricted to the cytoplasmic periphery of the NPC. This is achieved by covalent attachment of the ubiquitin-like protein SUMO-1 to the C-terminal domain of RanGAP, which targets the enzyme to the cytoplasmic face of the NPC [19–21].

RanBP1 and RanBP2

Binding of importins and exportins to RanGTP inhibits RanGAP-induced GTP hydrolysis by Ran [12, 22, 23]. This inhibition appears to be due to mutually exclusive binding of importins or exportins and RanGAP to partially overlapping sites on Ran [24]. Furthermore, RanGTP-importin complexes are stable, with half-lives of several hours [23, 25]. The binding of importins and exportins to RanGTP and exclusion of RanGAP would ordinarily make it impossible for transport receptors and Ran to disengage from each other and carry out repeated rounds of transport. Dissociation of RanGTP from these transport receptors is promoted by another family of Ran-binding proteins, represented by RanBP1 and RanBP2/Nup358. RanBP1 is 23 kDa and contains a single Ran binding site [26–29]; RanBP2 is 356 kDa and contains four Ran binding domains homologous to the RanBP1 Ran binding domain [30, 31]. RanBP1 or isolated Ran binding domains of RanBP2 bind RanGTP, but rather than inhibiting the GTPase-like importins, they stimulate GTPase activity by about an order of magnitude [24]. Furthermore, RanBP1 can form heterotrimeric complexes with RanGTP and importins. Binding of RanBP1 destabilizes the RanGTP/transport receptor complexes and thereby renders RanGTP in the complex accessible to RanGAP. Thus, in effect, RanBP1 removes RanGTP from importin β-like transport receptors, allowing RanGAP to activate Ran‘s GTPase [24]. While RanBP1 is a soluble protein, RanBP2/Nup358 is a major component of the of the NPC in higher eukaryotes [32]. Since *Saccharomyces cerevisiae* lacks RanBP2, he RanBP1 homologue Yrb1p handles all of the RanGTP-dissociating duty; indeed, deletion of *YRB1* is lethal [33].

NTF2
The continuous outflow of exportin-cargo complexes and cargo-free importins from the nucleus would lead to depletion of nuclear RanGTP and eventual shutdown of all nuclear transport, thus necessitating that Ran be restored to the nucleus. The return of Ran is mediated by another transport factor, NTF2 [34, 35]. NTF2 is unrelated to the importin β family, and it binds only RanGDP. Upon entering the nucleus, NTF2 and RanGDP must dissociate from each other, and Ran's GDP must be replaced with GTP. Both steps are fostered by a Ran-specific guanine nucleotide exchange factor (RanGEF) present in the nucleus.

RanGEF
The RanGEF, RCC1 (metazoans) and Prp20p (yeast), stimulates dissociation of Ran-bound nucleotide (either GDP or GTP) and the subsequent exchange reaction by stabilizing the nucleotide-free form of Ran. In vivo, Ran binds GTP, which is present in high concentration in the cell [16, 17, 36–38]. As required for maintenance of the asymmetrical distribution of RanGTP, RanGEF is restricted to the nucleus and bound to chromatin [39, 40]. Recent data show that RCC1 is actually a mobile enzyme that can cycle on and off chromatin [41]. Although RCC1 is highly active as isolated protein in vitro, chromatin association appears to be coupled to nucleotide exchange on Ran in living cells, which might provide an additional mechanism to ensure generation of RanGTP in the vicinity of chromosomes. Actually, RanGTP can be considered as a marker for the identity of the nuclear compartment or, more precisely, chromatin localization. The RanGTP halo around chromatin is used to provide a spatial cue in processes like mitotic spindle formation and nuclear envelope assembly following mitosis in higher eukaryotes [42–44].

Mog1p
Mog1p is a Ran-binding protein isolated as a multicopy suppressor of temperature-sensitive (ts) mutations in *S. cerevisiae* Ran, *GSP1* [45]. Mog1p is an evolutionarily conserved nuclear protein [46, 47]; it binds to both RanGTP and RanGDP, and competes with NTF2 for binding to RanGDP, even though Mog1p and NTF2 bind different sites in Ran [48]. Deletion of Mog1p in *S. cerevisiae* causes ts growth and defects in nuclear protein import but not in export of poly(A) RNA; similarly, mutations that prevent interaction between Mog1p and Ran cause the same phenotypes [49]. Deletion of Mog1p in *Schizosaccharomyces pombe* is lethal, while Mog1p ts mutants show defects in both protein import and poly(A) RNA export [47]. Overexpression of *NTF2* or *GSP1* suppresses the effects of *MOG1* deletion in *S. cerevisiae* [45], while overexpression of Ran suppresses *S. pombe MOG1* mutant phenotypes [47]. Mog1p stimulates GTP release from Ran and forms a stable complex with nucleotide-free Ran [50, 51]. Consistent with this activity, mutations in *MOG1* are synthetically lethal with *PRP20* (RanGEF) [42]. These observations coupled with the genetic interaction between *NTF2* and *MOG1* suggest that Mog1p plays a role in release of RanGDP from NTF2 and in subsequent exchange of GDP for GTP promoted by RanGEF [52].

Nuclear transport mechanism

Structure and composition of the NPC
The NPC is the only site of bidirectional exchange between the cytoplasm and the nucleus (for review see [53]). In contrast to transport systems of other organelles, the NPC does not constitute a diffusion barrier for small molecules such as ions or metabolites, which can freely partition between the nuclear and cytoplasmic compartments. Moreover, proteins do not have to unfold to be transported through the NPC, although large ribonucleoprotein complexes likely undergo partial unraveling during transport (e. g. see [54]).
The three-dimensional structure of the NPC has been obtained by analysis of electron microscopic images. Viewed along the axis of its central channel, the NPC exhibits an octagonal symmetry. The main mass of the pore complex forms a cylindrical structure, composed of spoke-ring complexes sandwiched between nuclear and cytoplasmic ring structures that are embedded in the nuclear envelope. The NPC is asymmetric with respect to its cytoplasmic and nuclear extensions. Attached to the cytoplasmic face are flexible filaments protruding into the cytoplasm, while fibrils emanating on the nuclear side converge at their ends to form a cagelike structure referred to as the nuclear basket. The vertebrate NPC is considerably larger than the yeast NPC, with an estimated molecular mass of 125 MDa for vertebrates compared to about 55–72 MDa for yeast [55–57].
Recent proteomic analysis of the composition of the NPC in both yeast and vertebrates has revealed that each NPC consists of about only 30 different proteins, the so-called nucleoporins, many of which are present at a copy number of eight or multiples of eight, reflecting the eightfold symmetrical arrangement of the NPC [58, 59]. It can be estimated that a mammalian pore consists of a minimum number of 400 individual proteins.
Nucleoporins have been localized within the NPC by immunogold-labeling techniques. Surprisingly, most yeast nucleoporins showed a very symmetric distribution on both entrances of the central channel, suggesting that in yeast the central part of the NPC displays twofold symmetry along its midplane axis. At least 9 nucleoporins showed either a partially or exclusively asymmetric distribution [58]. In higher eukaryotes, many of the

presently analyzed nucleoporins have been localized specifically to either one or the other side of the NPC [53]. Molecular asymmetry at the outside of the pore might be linked to mechanisms underlying the nucleocytoplasmic transport process (see below).

Based on their amino acid sequences, about two-thirds of the 30 different nucleoporins are reasonably conserved between yeast and mammals, although conservation is not as striking as for the soluble transport machinery [53, 59]. However, both types of NPCs are structurally and functionally similar. One important feature common to many nucleoporins from all species is that they contain numerous repeats of the amino acid sequences FG, FxFG and GLFG. These FG repeats provide binding sites for nuclear transport receptors such as importins and exportins, NTF2 and others [60–62]. It has been estimated that there are more than 1000 copies of such repeats within one NPC, and that their local concentration in the interior of the central channel could approach 50 mM [63]. This high concentration of FxFG repeats, of course, favors complex formation between individual repeats and nuclear transport receptors. To allow reasonable rates of transport, however, the interaction between transport receptors and FG repeats should be weak. Indeed, dissociation constants have been estimated to be in the micromolar range [63, 64].

Translocation through the NPC

It is an inherent feature of the NPC to permit the passive diffusion of small molecules but at the same time to restrict macromolecular movement between the cytoplasmic and nuclear compartments. Diffusion becomes inefficient if the molecular weight of the diffusive species approaches 20–40 kDa. The diffusion barrier is made by a single transport 'gate' located in the central domain of the transporter and has been estimated to have a mean diameter of 10 nm. At the same time, the NPC allows rapid translocation of cargo having appropriate signals that interact with nuclear transport receptors. These substrates can be as large as ribosomal particles and viral capsids (~35 nm diameter) [65]. It has been calculated that active transport allows a maximum mass flow of up to 80 MDa/per NPC/per second [64]. This rate translates to ~800 individual translocation events of a 100 kDa protein through one NPC every second. A central question in the field is to understand how this high rate of transport is brought about.

Studies have shown that the translocation of empty transport receptors, or small transport receptor cargo complexes, through the NPC occurs efficiently in the absence of energy provided by NTP hydrolysis [66–68]. Therefore, it is commonly accepted that translocation of simple transport substrates through the NPC is an energy-independent process. Since translocation relies on the interaction between the transport receptors and the FG-containing nucleoporins and is energy independent, nuclear pore passage can be best described as a process of facilitated diffusion. By definition, this step must be fully reversible. But how do transport receptor-nucleoporin interactions facilitate transport through the NPC? The problem of understanding how this works is twofold. Any model must, on the one hand, account for the speed and selectivity of the process. At the same time, mechanisms must explain how facilitated transport of cargo can occur against gradients of their chemical activities.

Several models have been put forth to explain how receptor-nucleoporin interactions promote selective transport (fig. 2). Rout et al. suggested the ‚Brownian affinity gate model‘ based on the observation that most nucleoporins are located on both the cytoplasmic and nucleoplasmic faces of the yeast NPC, where they could provide initial docking sites for transport receptors [58]. Thus, interactions between transport receptors and FG repeats present in the cytoplasmic and nucleoplasmic protrusions of the pore would increase the residence time of transport complexes at the entrances to the central gated channel. This trapping would raise the probability of complexes entering the channel. Passage through the pore is suggested to be by Brownian motion. Inert molecules would be rejected, since they would be much less likely to come into close proximity to the pore entrance. If by chance such molecules did reach the entrance, they might pass through the pore, but only if their diameters were below the exclusion limit of the central channel. Although this model is attractive at first glance, it does not explain the movement of large cargos through a narrow translocation channel. Moreover, docking sites at the cytoplasmic side of the pore do not appear to be required for efficient and selective protein import; it has been demonstrated that at least the cytoplasmic filaments and components of the cytoplasmic rings are dispensable [32].

Another model, which could account for movement of both large and small substrates and which does not rely on the existence of docking sites at the pore entrances, is the ‚selective phase model‘ of Ribbeck and Görlich [64]. This model predicts the existence of a meshwork of nucleoporins in the interior of the central channel formed by loosely folded nucleoporins interacting via their FG repeats. Both the size of the holes in the meshwork and its hydrophobic nature would determine the exclusion limit of the NPC by acting as a sieve and a selective phase. Transport receptors (together with bound cargo) can traverse this sieve because they are able to interact with the FG repeats, break their interactions and partition into the mesh. Inert cargos presumably are rejected by both size and by hydrophobic exclusion [69]. If one assumes that the central channel of the NPC is completely filled with this selective phase and can be as wide as 40 nm, this model could explain transport of large cargo. However, the existence of such a meshwork has not been proven.

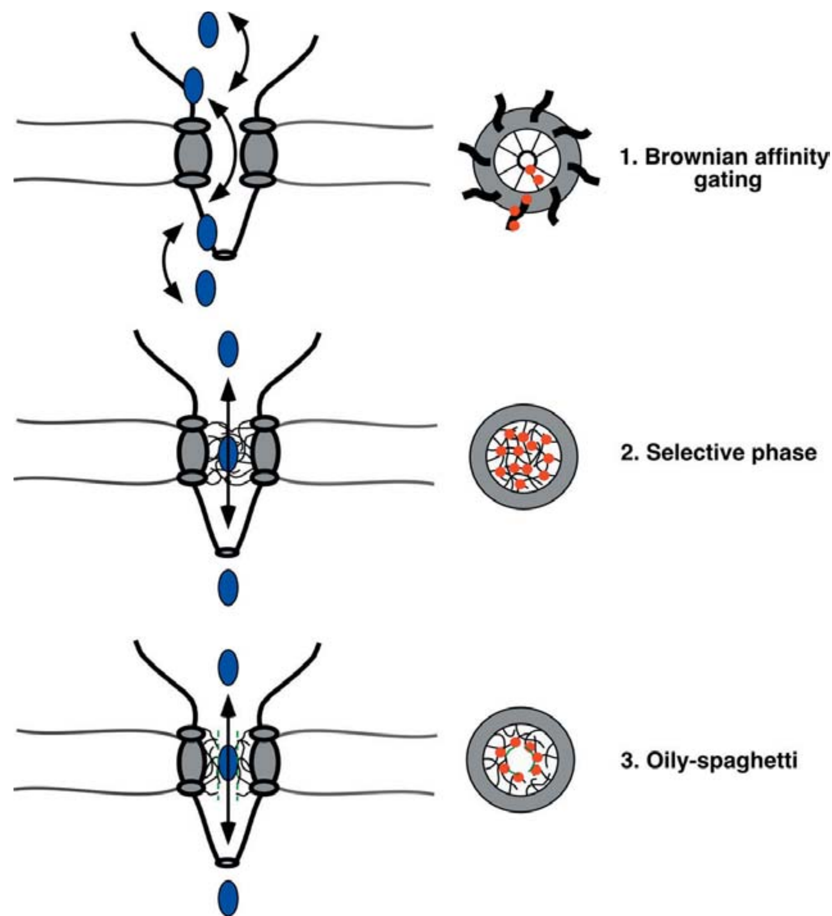

Figure 2. Models of translocation through the NPC. (1) The ‘Brownian affinity gate’ model [58] implies that a transport receptor/substrate complex (blue) initially binds to the cytoplasmic filaments of the NPC. It has been postulated that there exists a high abundance of FxFG-containing binding sites on the cytoplasmic and nuclear extensions of the NPC based on positional mapping of individual yeast nucleoporins (indicated as red dots depicted in the top view drawing on the right). The binding of the transport receptor/substrate complex in the proximity of the central channel of the NPC increases the likelihood of entrance of the complex into the interior of the NPC (affinity gating). Transfer through the channel would be by Brownian motion. (2) The ‘selective phase’ model [64] predicts the existence of a flexible meshwork of nucleoporins that interact with each other through their FxFG repeats. The meshwork is positioned centrally within the NPC and forms a permeability barrier for inert cargo. The interactions between nucleoporins in the mesh can be broken transiently by nuclear transport receptors, which can partition into the lattice by engaging into hydrophobic interactions with FxFG repeats. (3) According to the ‘oily-spaghetti’ model [70] the NPC is an open structure with a central channel width of 10 nm. Flexible FxFG-containing nucleoporins coat the wall of this channel and provide binding sites for the passage of nuclear transport receptors. Transport receptors can push aside the loose nucleoporin chains during passage. Transient association with FxFG repeats and random motion would achieve translocation.

A third hypothesis, the ‘oily spaghetti model’ of Macara, combines features of the selective phase model with existence of a central open pore of 10 nm diameter [70]. Nucleoporins exposing their FG repeats would make up a flexible coat filling the tube walls that could allow partitioning of receptor-cargo complexes, as proposed in the selective phase model. Transient association with FG motifs and random motion would achieve translocation. The nucleoporins coating the interior of the NPC are imagined to be flexible enough to also allow the passage of large cargo.

All of the forgoing models assume that translocation occurs by random or facilitated diffusion. This brings us to the second part of the problem. How can a fully reversible process like diffusion be converted into directional movement? Directionality could be provided by coupling facilitated translocation through the pore to substrate dissociation from the transport receptor in the target compartment. As indicated before, for the majority of transport pathways, the RanGTPase system is instrumental in transport ([3] for review). The RanGTP gradient across the NPC determines the vectorial nature of transport, since the high nuclear RanGTP concentration ensures rapid cargo dissociation from importins in the nucleus, while RanGAP, in conjunction with RanBP1/RanBP2, brings about rapid dissociation of export complexes in the cytoplasm.

If the RanGTP gradient provides only directional information and translocation through the central channel is

not an energy-driven process, then one prediction is that inversion of the RanGTP gradient would in turn reverse the direction of transport. Indeed, it has been shown that a high concentration of cytoplasmic RanGTP can invert export and drive exportin/substrate complexes into the nucleus [71]. This also implies that the NPC itself provides no major directional cue to the transport reaction. Thus, it is the coupling of facilitated transport to the RanGTP gradient that determines directionality and allows accumulation of cargo against a gradient of its own chemical activity. Another factor that may contribute to accumulation of cargo against their activity gradients is that during translocation through the NPC, cargo is bound to receptors; thus, the chemical activity of translocating cargo is not equivalent to the chemical activity of the cargo once it is released into the target compartment and engages in other interactions.

In theory, the magnitude of cargo accumulation is determined by the steepness of the primary RanGTP gradient. The ratio between the concentration of free nuclear and cytoplasmic RanGTP is at least 200-fold [72, 73]. This value sets an upper limit for substrate accumulation, i.e. at 100% efficiency of coupling between translocation and the RanGTP gradient, provided there are no other states of cargo such as binding to additional retention sites. But what determines the coupling efficiency? Coupling efficiency appears to be inversely proportional to the rate of diffusion of cargo through the NPC [74]. For example, biotinylated GST chimeras containing nuclear export signals were injected in the cytoplasm of *Xenopus* oocytes. Although these fusion proteins contained export signals only, they efficiently accumulated in the nucleus by receptor-mediated ‚reverse' export against the native RanGTP gradient (i.e. imperfect coupling). Reverse export could be measured because the biotinylated substrates were artificially trapped in the nucleus by streptavidin. The rates of nuclear import of these substrates were almost comparable to their natural export rates. For large cargos unable to diffuse through the NPC permeability barrier, reverse transport was negligible. Thus, reverse export complexes must have been formed within the NPC. It can be concluded that there exists a steep drop in the RanGTP concentration at the cytoplasmic face of the pore so as to prevent formation of (unfavorable) reverse export complexes at the cytoplasmic entrance, and at the same time, there is a high concentration of RanGTP/transport receptor complexes within the NPC that must be accessible to diffusible cargo.

If transport of diffusible sized cargo is less than fully coupled to the RanGTP gradient, one way to optimize coupling for small cargo might be to link the dissociation of transport receptor/cargo complexes directly to their exit from the translocation site, hence preventing reverse translocation of cargo on the spot. The fact that transport receptors often display high-affinity, RanGTP-sensitive interactions with nucleoporins at terminal translocation sites may reflect such a mechanism. A model incorporating the importance of terminal transport receptor binding sites at the cytoplasmic filaments and nuclear baskets is the 'affinity gradient model' of Ben-Efraim and Gerace [75]. This model suggests that a gradient of increasing affinities of transport receptor binding sites along the translocation pathway through the pore contributes to the directional movement of transport receptors. Although, as discussed before, the RanGTP gradient is sufficient to determine the directionality of transport, the trapping at terminal binding sites could be used to couple exit from the translocation site directly to the disassembly of transport complexes.

One example is the disassembly of export complexes at the cytoplasmic filaments in higher eukaryotes. A main constituent of these filaments is the nucleoporin RanBP2/Nup358 ([32] and reference therein). RanBP2/Nup358 possesses both FG-repeat motifs for transport receptor association as well as four RanGTP binding domains of the RanBP1 type that are known to assist in disassembly of RanGTP/transport receptor complexes. In addition, RanGAP is associated with RanBP2/Nup358. Hence, RanBP2 provides a perfect molecular platform for the absorption of factors needed for the efficient disassembly of export complexes. The RanBP1-like domains of RanBP2 are expected to pick up RanGTP from export complexes and present them to RanGAP, leading to disassembly of the emerging export complex. There is, however, no homologue of RanBP2 in yeast, and yeast RanGAP is distributed throughout the cytoplasm. Thus, transport complex disassembly directly at the NPC might be helpful but is apparently not a requirement for the transport process. Similarly, the nucleoplasmic face of the NPC provides high-affinity binding sites for certain import receptors. While nucleoplasmic nucleoporins such as Nup153 are, for example, not required for protein import mediated by transportin (a member of the importin β family), the importin β-dependent accumulation of NLS substrates is significantly reduced in nuclei lacking Nup153 and additional nucleoporins of the nuclear basket [76]. This observation correlates well with the fact that Nup153 provides a strong terminal binding site in importin β/importin α-mediated NLS import. This site has been implicated in the RanGTP-dependent dissociation of NLS import complexes. Therefore, importin β-mediated NLS import might serve as one example in which these terminal sites are required for efficient import and for the coupling of translocation to dissociation. In the absence of a Nup153 trap, coupling would be less efficient. Consequently, net import of NLS substrates might not be observed because the import complex may be reexported rapidly.

Taken together, it should be apparent that importin/exportin-mediated nuclear transport does not utilize meta-

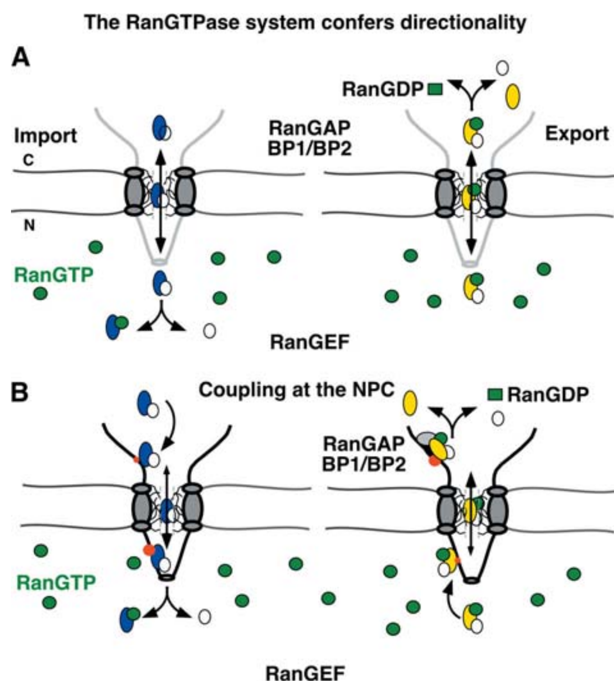

Figure 3. Directionality of transport. The RanGTP gradient determines the vectorial transport of cargo. Translocation through the NPC is an energy-independent, reversible reaction. To achieve directionality of substrate transport, translocation is coupled to a second reaction (*A*). During import, this reaction is the dissociation of the import complex by RanGTP, and the subsequent binding of the import substrate to nuclear partners. The export process gains directionality by export complex disassembly in the cytoplasm involving GTP hydrolysis on Ran mediated by RanGAP. Coupling of the secondary reactions directly to NPC exit (*B*) should increase the efficiency of the overall process. High-affinity interactions between terminal nucleoporins (depicted as large red dots) and transport receptors help to ensure that dissociation of transport complexes occurs before reverse transport can happen. A gradient of increasing binding affinities for transport receptors to nucleoporins across the NPC has been measured and led to the suggestion of the ‚affinity gradient' model [75]. Note that the affinity gradient may only have a minor contribution on the overall directionality of transport, which is primarily determined by the RanGTP gradient.

bolic energy as provided by nucleotide triphosphate hydrolysis directly for the movement of carrier-cargo complexes through the NPC. Rather, the requirement for metabolic energy in transport lies in creating an asymmetrical distribution of RanGTP across the nuclear envelope (see fig. 3).

Nuclear Transport Receptors

Different classes of shuttling transport receptors mediate nucleocytoplasmic transport. These receptors are defined by their ability to directly contact FG-containing nucleoporins, thereby facilitating the passage of cargo molecules through the NPC. The largest class of nuclear transport receptors is the superfamily of importin β-related proteins referred to as importins and exportins or karyopherins [77]. A second group of transport receptors comprises fewer constituents, all of which share similarity to the founding member, NTF2. As indicated above, NTF2 mediates import of RanGDP into the nucleus [34, 35]. Related to NTF2 are p15 and its homologues [78]. p15, a cofactor in messenger RNA (mRNA) export, and NTF2 are both small proteins with striking structural resemblance. The similarity in their tertiary structure is even more prominent than their primary sequence homology [61]. It seems to be a common feature of these small proteins either to form homodimers, as is the case for NTF2, or to engage into heterodimeric complexes with proteins harboring domains having a similar fold. p15, for example, forms heterodimers with members of a third group of transport receptors that includes proteins related to the mRNA export receptor TAP (see below and [79]).

Importin β-like transport receptors account for the majority of cargo flow through the NPC. There are 14 members of this family in yeast and more than 20 in higher eukaryotes (tables 1 and 2). All members of the importin β superfamily contain an N-terminal RanGTP binding domain, have a similar overall structural organization, possess an acidic isoelectric point, and range in their molecular weight from 90 to 145 kDa. As outlined before, cargo binding to these receptors and, hence, their direction of transport, is controlled by association with RanGTP. Most importin β-like receptors appear specialized to confer transport in only one direction. A few such receptors can mediate both import and export (see tables 1 and 2).

Just how RanGTP regulates substrate binding to importins and exportins in an opposing manner is not yet fully understood. So far, structural information has been obtained exclusively for importins, i.e. importin β and transportin [60, 80–82]. These proteins consist of a consecutive arrangement of so-called HEAT repeats, which are also present in exportins. HEAT repeats derive their name from non-family members such as **h**untingtin, **e**longation factor 3, the **A** subunit of protein phosphatase 2A and **T**OR1 [83]. HEAT repeats are about 40 amino acids in size and form two α helices connected by a short turn. In importin β-like transport receptors, successive HEAT repeats are joined by short helices or linkers and fold into a spiral-like right-handed helical structure composed of two arches representing the N-terminal and C-terminal halves of the receptors [80–82]. Contacts to FG-containing nucleoporins occur from the outside of the spiral-shaped receptor molecule. One FxFG interaction site has been resolved at the surface of the N-terminal domain of importin β [60]. Overall, these transport receptors appear to be flexible molecules, which might stretch and compress their domains by twisting. RanGTP can bind in the inner surface of the N-terminal arched half-pipe. The mode of Ran binding to the N-terminal arch might be conserved throughout the family. Importins have a high affinity for RanGTP, implying that their N-terminal arch might always be freely accessible to RanGTP [84]. Ex-

Table 1. Mammalian RanGTP-binding transport receptors.

Names mammals (alternative names)	Accession number[a]	Adaptors	Transport substrates	Function	References
Importin β (Kapβ1, p97, PTAC97)	NP_002256		proteins containing basic stretches such as: ribosomal proteins; HIV Rev and Tat, HTLV Rex; cyclin B, core histones and others; histone H1 and ribosomal proteins in conjunction with Importin 7	import	[96, 249–252]
		importin α family	NLS proteins	import	[5, 7, 253–255]
		snurportin 1	U snRNPs	import	[256]
		XRIPα	replication protein A	import	[257]
Transportin 1 (Kapβ2a, TRN1)	NP_002261		mRNA binding proteins, ribosomal proteins	import	[96, 258]
Transportin 2 (Kapβ2b, TRN2)	NP_038461		overlapping function with transportin 1, suggested to be involved in mRNA export	import export?	[259] [219, 220]
Transportin SR1[b] (TRN-SR1)	AAD38537		SR proteins	import	[260]
Transportin SR2 (TRN-SR2)	NP_036602		SR proteins containing phosphorylated RS domains	import	[261]
Importin 4a	AAL55522		ribosomal protein S3a	import	[98]
Importin 4b	NP_078934		?	import	[98]
Importin 5 (Kapβ3)	CAA70103		Ribosomal proteins, core histones and other basic proteins	import	[96, 262, 263]
Importin 7	NP_006382		ribosomal proteins, core histones and other basic proteins; histone H1 and ribosomal proteins in conjunction with importin β	import	[96, 249]
Importin 8	NP_006381		SRP19	import	[264]
Importin 9	NP_060555		core histones with a preference for H2B, import of ribosomal proteins	import	[93, 98]
Importin 11	AAF21936		UbcM2, ribosomal protein L12	import	[265, 266]
Importin 13	NP_055467		UBC9, Y14 eIF1A	import export	[267]
CRM1/Exportin 1 (XPO1)	NP_003391		proteins with leucine-rich NES sequences, snurportin 1	export	[85, 111, 268, 269]
		PHAX	NES-containing adaptor for U snRNAs	export	[116]
		HIV Rev	NES-containing adaptor for unspliced HIV-RNAs	export	[85, 108, 270]
		NMD3	potential NES-containing adaptor for pre-60S ribosomal subunits	export	[309, 310]
CAS	NP_001307		importin α family members	export	[14]
Exportin-t (Xpo-t)	NP_009166		tRNAs	export	[86, 87]
Exportin 4	NP_065252		eIF5A	export	[271]
Exportin 5	NP_065801		tRNA and perhaps other structured RNAs; ILF3 and eEF1A (RNA-mediated binding)	export	[135, 136, 272]
RanBP6	AAC14260		?	?	database
RanBP16	NP_055839		?	?	[273, 274]
RanBP17	NP_075048		?	?	[273, 274]
RanBP20	NP_083092		?	?	database

[a] Human or mouse.
[b] Potential splice variant of TRN-SR2.

Table 2. *S. cerevisiae* RanGTP-binding transport receptors.

Names – yeast (alternative names)	Accession number[a]	Closest manmalian homologue	Yeast transport substrates	Function	References
Kap95p Rsl1p, Impβ	AAB67265	importin β	NLS proteins in conjunction with Kap60p (Srp1p)	import	[275]
Kap104p	CAA84959	transportin	mRNA binding proteins Nab2p and Hrp1p	import	[276]
Mtr10p Kap111p	CAA99366	transportin SR, importin 13	mRNA binding Npl3p	import	[99]
Yrb4p Kap123p	AAC03208	importin 4	ribosomal proteins, core histones H3 and H4, SRP proteins	import	[95, 97, 277, 278]
Pse1p Kap121p	CAA89141	importin 5, RanBP6	ribosomal proteins and other proteins with basic stretches (e.g.Yra1, Spo12p, Ste12p, Yap1p, Pho4p), core histones, SRP proteins	import	[95, 97, 278–283]
Nmd5p Kap119p	CAA89663	importin 7, importin 8	ribosomal proteins, core histones, Hog1p, Crz1p, Dst1p and other basic proteins	import	[284–286]
Sxm1p Kap108p	AAB64837	importin 7, importin 8	Lhp1p and ribosomal proteins	import	[287]
Pdr6p Kap122p	CAA96716	importin 13, transportin SR	Toa1p and Toa2p	import	[288, 289]
Kap114p HRC1004	CAA96960	importin 9	TBP, core histones with a preference for H2B and H2A	import	[94, 100]
Lph2p Kap120p	AAB68237	importin 11	ribosmal subunit export or ribosomal protein import	?	[234]
Msn5p Kap142p	AAB64771	exportin 5	replication protein A Pho4p, Crz1p, Mig1p, Ste5p, Cdh1p	import export	[290] [291–296]
Crm1p/Xpo1p Kap124p	CAA97246.	CRM1/ XPO1	proteins with leucine-rich NES sequences, including Nmd3p (potential NES-containing adaptor for pre-60S ribosomal subunits)	export	[150, 229, 230]
Cse1p Kap109p	CAA96957	CAS	Kap60 (Srp1p)	export	[297–299]
Los1p Kap127p	CAA82050	exportin-t	tRNAs	export	[124]

portins, by contrast, display a high affinity for RanGTP only if also complexed with cargo [14, 85–87]. This suggests that the N-terminal arch in exportins may undergo a conformational change upon substrate binding to the C-terminal arch, such as, for instance, by twisting of the HEAT repeat helix. Since the binding of RanGTP and substrate to exportins is cooperative in most cases, RanGTP binding may have a similar influence on the C-terminal substrate binding pocket. The difference between importins and exportins must be in the coupling of RanGTP binding to the conformation or accessibility of the C-terminal arch. For importins, it has been suggested that upon binding of Ran to the N-terminal domain, an acidic loop centrally placed in the molecule transmits structural changes to the C-terminal substrate binding site [88]. However, we will only be able to obtain complete insight into the mechanism of RanGTP-regulated cargo binding to importins and exportins when the structure of one transport receptor is solved in both conformations.

Nuclear transport signals

Nuclear transport is a highly specific process that relies on the recognition of cargo by specific features present within the cargo molecules. These transport 'signals' can be recognized either by the importins and exportins directly or by adapter molecules. Different nuclear targeting signals have been identified that funnel cargos into the different transport routes (see table 3). Most transport signals do not fit a well-defined consensus, and for many no consensus has been advanced; rather, general features such as length, charge, or hydrophobicity and spacing of key residues are important. Prediction of these signals by computer programs is not always reliable since considerable deviations can occur.

Nuclear transport signals are required for the directional movement of a cargo across the NPC. Consequently, one might distinguish import from export signals. While most signals fit into this classification, some import signals have been suggested to possess export activity as well, so-called shuttling signals [89]. Reporter proteins fused to

Table 3. Signal sequences in nucleo-cytoplasmic transport.

Transport signal	Example substrates	Sequence	References	References	Transport receptor(s)
Classical monopartite NLS	SV40 T antigen	PKKKRKVE	[300]	[298]	Impβ/Impα
Classical bipartite NLS	nucleoplasmin	KRPAATKKAGQAKKKKLD	[301, 302]	[299, 300]	Impβ/Impα
M9 domain	hnRNPA1	YNDFGNYNNQSSNFGPMKGGNFGGRSSGPY	[303, 304]	[301, 302]	transportin
BIB domain	rpL23a	VHSHKKKKIRTSPTFRRPKTLRLRRQPKYPR KSAPRRNKLDHY	[96]	[94]	transportin, Imp5, Imp7, Impβ
RS domain	SR proteins	phosphorylated RS domains	[260, 261, 305]	[258, 259, 303]	transportin SR2
Leucine-rich NES	HIV Rev, PKI	consensus: L-X_{2-3}-(L,I,M,F,M)-X_{2-3}-L-X-(L,I,V)	[306]	[304]	CRM1

these signals are nuclear at steady state, indicative of the import activity of the signal. When analyzed in interspecies heterokaryon assays, these shuttling signals appear to confer receptor-mediated export from one (donor) nucleus and import into the (acceptor) nucleus. It should be noted, however, given the reversibility of translocation through the pore, that this export activity may, in some cases, simply reflect the capability of the signals to undergo a 'reverse' import reaction. In other words, the same import receptor that carried a cargo into one nucleus may have reexported that cargo to the cytoplasm, from where it could enter the other nucleus. Similarly, leucine-rich nuclear export substrates have been shown to act as import signals if able to penetrate the diffusion barrier of the NPC [74]. Hence, the heterokaryon assay should be interpreted with caution. The acceptor nucleus might work like a trap for cargo which exits the donor nucleus on the import route. The efficiency by which this reverse import might occur will depend on how well an import substrate is dissociated from its transport receptor by RanGTP in the nucleus, on the steepness of the RanGTP gradient, on the influence of nuclear retention and, by analogy to reverse export, perhaps also on the cargo size. Many of the shuttling signals identified so far may actually function, for the most part, in import.

Nuclear transport pathway

Nuclear Import

As mentioned, most receptor-mediated import pathways rely on importin β-like molecules. Importin β itself is not only the first identified transport receptor but the most versatile, as it recruits cargo in many different ways (see tables 1 and 2 and references therein). Most of its substrates contain classical, basic NLS recognized by an adaptor, importin α. Snurportin is another importin adaptor that binds the trimethylated cap structure of U-rich small nuclear RNAs (U snRNAs), thus further adding to the range of cargo which can be imported by importin β. In addition, importin β can directly bind and import proteins with extended basic stretches such as ribosomal proteins. Finally, importin β forms heterodimers not only with adaptor proteins but also with other importins. The importin β/importin 7 heterodimer drives nuclear import of the linker histone H1 and may also support import of particular ribosomal proteins.

Some import signals allow association with only a single transport receptor. The classical basic NLS, for example, is imported exclusively by importin β in conjunction with members of the importin α family. In higher eukaryotes, there exist several importin α isoforms, all of which recognize basic NLS [90, 91]. In the context of different substrate proteins, however, a given basic NLS may show a preference for one importin α family member [92].

Many other import signals are also basic in nature and often part of protein domains that bind RNA and DNA. Often, these signals can bind in vitro to different importins (see table 3), suggesting redundancy in nuclear import pathways. However, there is likely to be a preference for one particular transport receptor in vivo. Nuclear import of core histones is such an example of receptor preference. In vitro, nuclear import of H2A and H2B can be mediated by at least five different importins [93]. Analysis of importin gene mutations in yeast shows that this redundancy also exists in vivo [94]. However, while mutations in at least four different yeast import receptors affected nuclear import of the two histones, in cell extracts Kap114p was identified as the major binding partner of H2A and H2B and, hence, suggested to constitute the preferred import pathway in vivo. Still, why would Kap114p be selected as the major import receptor for H2A and H2B if, in principle, the other importins can serve in this role just as well? In the case of histones, an additional fac-

tor, the nucleosome assembly protein Nap1p, has been shown to confer specificity in importin association by promoting the specific association of the H2A and H2B NLS with Kap114p [95].

Redundancy in nuclear import pathways was also observed for other basic cargo substrates such as ribosomal proteins, which, as alluded to above, appear to be recognized by way of short, basic stretches of amino acids [96, 97]. Again, in the case of small basic proteins, why are there so many different importins that recognize them if one such signal together with an appropriate transport receptor would be sufficient? The answer may stem from how this redundancy might have evolved. Recently, an interesting suggestion has been offered based on the observation that importins fulfill a dual function – as nuclear import receptors and as cytoplasmic chaperones for exposed basic domains [98]. This chaperone task of the importins requires optimal shielding of exposed basic domains on nuclear proteins during their import. Since the basic domains in the numerous import substrates are quite diverse depending on their specific function in the nucleus, a number of different import receptors may have evolved to guarantee an optimal shielding of their charged surfaces to prevent aggregation before or during the import process.

Upon arrival in the nucleus, import substrates need to be dissociated from import receptors. Generally, this involves binding of RanGTP to the importin. However, RanGTP alone is not sufficient in all cases. Sometimes, intranuclear targeting to the correct binding partner contributes to substrate release from importins. In one scenario, the yeast RNA-binding protein Npl3p dissociates from its import receptor Mtr10p only upon incubation with both RanGTP and RNA [99]. Similarly, double-stranded, TATA-containing DNA stimulates RanGTP-mediated dissociation of TATA-binding protein (TBP) from Kap114p [100].

In other cases, specific cofactors stimulate dissociation of cargo from particular importins. A search for factors aiding the disassembly of the trimeric NLS/Impα/Impβ (Kap60p/Kap95p) complex revealed a function for the two yeast nucleoporins Nup1p and Nup2p. Both are located at the nucleoplasmic face of the NPC and, in vitro, accelerate the rate of import complex disassembly [101–103]. Cells lacking *NUP2* show specific defects in NLS import. It has been suggested that Nup2p contributes to the release of the NLS protein from the Kap60p/Kap95p heterodimer at the nucleoplasmic face of the NPC by displacing the NLS substrate from importin α, thereby coupling nuclear pore complex exit to import complex disassembly. Nup2p contains an N-terminal domain needed for the interaction with importin α, a central domain with FG motifs, and a C-terminal RanBP1-like RanGTP binding domain. Interestingly, Nup50 (Npap60), a mammalian protein displaying the same domain organization and distant homology to Nup2p, has been proposed to function in a slightly different manner [104–106]. In contrast to Nup2p, Nup50 does not appear to compete for binding of NLS to importin α [106]. Accordingly, Nup50 has been suggested to promote NLS import at a prior stage, such as import complex formation or nuclear pore passage, rather than having a primary role in import complex dissociation. Nup50 and, curiously, Nup2p are mobile proteins with the ability to shuttle to the cytoplasm, consistent with a role in nuclear import prior to cargo dissociation [106, 107].

Nuclear Export

CRM1-mediated export

Exportins facilitate the nuclear pore passage of a multitude of different proteins, RNA molecules and ribonucleoprotein particles (RNPs). The export receptor CRM1/Xpo1p is the most versatile of all export factors, being involved in export of many different classes of proteins such as cell cycle regulators, transcription factors, RNA binding proteins and numerous others. CRM1 binds its cargos in different modes. Most commonly, CRM1's export substrates contain a short, leucine-rich nuclear export signal that was first identified in proteins such as HIV Rev and protein kinase A inhibitor [108, 109]. Export complex formation with nuclear export signal (NES) substrates requires RanGTP binding to CRM1 [85]. It should be noted that not all leucine-rich NESs are exactly alike and individual, isolated NES segments are exported by CRM1 with different efficiencies [110] (for a consensus see table 3). The substrate known to bind CRM1 with highest affinity is snurportin, an importin β adaptor (see preceding section). However, the domain in snurportin required for CRM1 association does not resemble the consensus leucine-rich NES as it is larger and contains only a number of dispersed leucine residues [111].

CRM1 also contributes to nuclear export of different classes of cellular RNAs or ribonucleoproteins (RNPs), for instance U snRNAs, ribosomal RNAs, signal recognition particle (SRP) and certain mRNP complexes (see tables 1, 2 and [112–115]). Since CRM1 cannot bind RNA directly, recruitment of CRM1 to the individual RNPs has been suggested to involve distinct NES-containing adaptor proteins specifying the individual pathways. However, in most cases the proposed adaptors have so far remained elusive. For example, export of SRP from yeast cell nuclei requires functional Crm1p (Xpo1p) [113], but it is as yet unclear how CRM1 is recruited to export competent SRP.

Our understanding of how CRM1 guides the export of U snRNAs is the most advanced. In metazoans, a subset of spliceosomal U snRNAs are exported from the nucleus. Export depends upon recognition of the 7-methyl guano-

sine cap structure on the RNA by the nuclear cap binding complex (CBC) and upon the NES-containing protein PHAX (phosphorylated adaptor for RNA export) [116]. PHAX serves as a bridge between the CBC-bound U snRNA and CRM1/RanGTP. The stability of the export complex is regulated by the phosphorylation status of PHAX. In the nucleus, PHAX is phosphorylated, promoting export complex assembly. After nuclear export, PHAX is dephosphorylated in the cytoplasm, aiding export complex disassembly.

Similar to the role of NUP50/Nup2p as cofactors in the classical NLS import pathway, CRM1-mediated export of NES substrates also involves a nuclear cofactor, Yrb2p in yeast and RanBP3 in vertebrates, with a modular domain structure reminiscent of Nup2p [117–119]. In yeast, both overexpression and deletion of *YRB2* specifically impair NES-mediated protein export [120, 121]. Yrb2 and RanBP3 bind directly to CRM1 but not to other export receptors. The binding of RanBP3 to CRM1 stabilizes the CRM1/export substrate interaction, resulting in formation of a quaternary complex consisting of RanBP3, RanGTP, CRM1 and the export substrate [122, 123]. In light of the observation that many leucine-rich NES export substrates interact weakly with CRM1/RanGTP in vitro, a function of RanBP3/Yrb2p in the formation of a stable export complex is alluring. Consistently, RanBP3 stimulates NES protein export when analyzed in an in vitro nuclear transport assay [122, 123]. By analogy to the role of Nup50p in NLS import, RanBP3/Yrb2p might be a constituent of the NES export complex, possibly possessing a function beyond export complex formation in the nucleus. This conjecture would imply that RanBP3/Yrb2p shuttles between the nucleus and the cytoplasm. However, shuttling of Yrb2p in yeast and RanBP3 in *Xenopus* oocytes could not be observed ([121] and N. Treichel and U. Kutay, unpublished results). In contrast, the analysis of RanBP3 export in interspecies heterokaryons suggested that RanBP3 shuttles between the nucleus and the cytoplasm [123]. Since RanBP3 shuttling in this assay was sensitive to leptomycin B (which inhibits CRM1‘s activity), these data are consistent with a model of RanBP3 accompanying CRM1 to the cytoplasm.

tRNA nuclear export

An importin *β*/karyopherin family member identified as a transfer RNA (tRNA) export receptor is exportin-t/Los1p ([86, 87, 124] see also recent reviews by [125] and [126]). Virtually unique among RNA export receptors, Exp-t and Los1p bind directly to tRNA without intervention of adaptor proteins and, like all importin *β*/karyopherin export receptors, Exp-t/Los1p bind cargo and RanGTP cooperatively. In oocyte injection experiments, tRNAs are exported more rapidly than other injected RNAs. This faster kinetics may be due in part to direct binding of tRNAs to Exp-t and independence from having to assemble a larger export complex. Also, Exp-t contains two NPC domains that bind to peripheral nucleoporins; the N-terminus binds to both Nup153 (on the nuclear side of the NPC) and RanBP2/Nup358 (cytoplasmic side) in a RanGTP-dependent manner, while the C terminus binds to CAN/Nup214 (cytoplasmic side) in the absence of RanGTP. It has been suggested that these interactions with peripheral NPC components increase the rapidity of Exp-t‘s nuclear export and reentry cycle [127].

Exp-t binds most efficiently to tRNAs with mature 5′ and 3′ ends, including 3′ end addition of the CCA nucleotides, and more efficiently to tRNAs with base modifications compared with in vitro synthesized, mature yet unmodified tRNA. Although the differences in affinities between mature and precursor tRNAs are not very large (~5–10-fold), tRNAs with incompletely processed ends or with mutations that alter their three-dimensional structure are exported inefficiently in oocyte injection experiments [87, 128–131]. This preference for processed, structurally intact tRNAs suggests a mechanism ensuring that only functionally mature tRNAs are delivered to the cytoplasm. However, Exp-t does not discriminate between unspliced and spliced tRNAs, and provided there is excess Exp-t, unspliced tRNAs can be exported [129, 130]. The requirement for an excess of Exp-t to transport unspliced tRNA suggests that unspliced tRNAs are normally retained in the nucleus by other proteins. Also, in oocytes, tRNA end processing occurs after splicing, further ensuring that unspliced tRNAs do not prematurely access the Exp-t pathway [131]. The tRNA-binding properties of Los1p have not been systematically defined, although they are assumed to be the same as Exp-t. If so, there must be a different mechanism that prevents Los1p-mediated export of unspliced tRNAs, since end processing usually precedes splicing in yeast.

Although previously assumed to be a cytoplasmic-only process, aminoacylation of tRNAs also appears to occur in the nucleus in both animal cells [131] and yeast [132, 133]. While not essential for nuclear exit of tRNA [129, 130], nuclear aminoacylation has been suggested to provide an additional proofreading step to ensure that only functional tRNAs are exported [131]. It is not yet clear how aminoacylation contributes to more efficient export. Exp-t binds non-aa-tRNAs with high affinity [87, 130], and it is unknown whether aminoacylation increases the affinity of substrate binding still further. Nonetheless, since cells containing inactivating mutations in both Los1p and any of several aminoacyl-tRNA synthetases did not show any greater growth defect than cells with only one mutation, it appears that nuclear aminoacylation and Los1p operate in the same export pathway [134].

Alternative tRNA export pathways

In oocyte injection experiments, inhibition of Exp-t by specific antibodies reduced tRNA export by only ~80%

[129, 130]. Thus, while Exp-t is the major tRNA export pathway, one or more additional export routes remained a possibility. Most recently, another importin family member, exportin 5 (Exp5), has been found to export tRNAs [135]. Like Exp-t, Exp5 binds directly to tRNAs in a RanGTP-dependent manner [135, 136]. Qualitatively, Exp5 and Exp-t appear to bind different (but perhaps overlapping) subsets of tRNAs, and Exp5 seems to have an overall lower affinity for tRNA. Exp5 binds aminoacylated (aa) and non-aa-tRNAs. While the precise structural requirements for tRNA binding to Exp5 are not yet known, Exp5 and Exp-t interact with tRNAs differently, since eEF1A (which delivers aa-tRNAs to the ribosome) can bind via tRNA to Exp5 but not to Exp-t. This difference is understandable because both Exp-t and eEF1A both bind the tRNA acceptor stem and CCA. Finally, while Exp-t binds only tRNA, Exp5 may export other RNAs that contain stable mini-helices [135–137].

In yeast, deletion of the *LOS1* gene causes no significant growth phenotype and some tRNAs are affected little, if any, in their nuclear export [133, 138, 139]. While this situation may indicate the presence of one or more parallel tRNA export pathways, the reality of an additional export mechanism remains unsettled. Deletion of Msn5p, the yeast homologue of Exp5, causes no obvious tRNA export deficiency [140]. Perhaps this is not surprising, given that Exp5 probably constitutes a relatively small fraction of the total tRNA export activity in animal cells; it will be interesting to examine the yet unreported phenotype of a *los1 msn5* double mutant. Then again, Msn5p is known to export phosphorylated proteins [141] and, thus, may not be a true Exp5 orthologue. Also, a number of tRNA-interacting proteins, including eEF1A, Cca1p (which adds the 3′ CCA), and Mes1p (methionyl-tRNA synthetase) can act as multicopy suppressors of the export defects for those tRNAs that are affected in *LOS1*-deleted cells [133, 140]. Thus, these other factors have been suggested to be part of one or more Los1p-independent tRNA export pathways. Cca1p could possibly form a stable complex with tRNA (see [142]), and yeast Cca1p shuttles between the nucleus and the cytoplasm [140], making it an attractive candidate for an adaptor that mediates an alternative export pathway (Cca1p has not been found to interact with any nucleoporin, making it unlikely to be an export factor itself; [140]). Such an alternative pathway probably does not utilize a member of the importin β family since, except for Los1p, mutations in all known yeast exportins (and some importins as well), failed to affect tRNA export [140]. A *los1 cca1* double mutant did not show a stronger phenotype than either single mutant, but perhaps this is because any number of aa-tRNA synthetases can provide alternate export pathways. Still, tRNAs are small (~7.5 nm in the longest dimension) and may diffuse through the NPC, although diffusion is expected to be slow. Given that eEF1A, Cca1p and Mes1p are all found wholly or partly in the cytoplasm, overexpression of these proteins may drive tRNA diffusion toward the cytoplasm by increasing the available cytoplasmic binding sites.

mRNA nuclear export

The large size and sequence complexity of mRNA molecules, especially their intron-containing precursors, requires that transcription, pre-mRNA processing and nuclear export of mRNA proceed in an orderly fashion to increase the efficiency of each step while minimizing and detecting errors. This requirement is best met when communication exists between the various stages in mRNA biogenesis. In fact, not only the nuclear stages but subsequent cytoplasmic phases in the life of an mRNA, i.e. subcellular localization, translation and degradation, are each mechanistically linked (see reviews by [143–149] and discussion below). From start to finish, this coupling is achieved at the molecular level by an extensive assortment of RNA-binding proteins and other trans-acting factors, such as the cap-binding complex, the spliceosome, SR proteins and other splicing factors, hnRNP proteins, 3′ UTR-binding proteins, the poly(A)-binding protein and a number of newly recognized factors involved in mRNA export. Unlike other RNAs, nuclear export of the majority of cellular mRNA relies neither on members of the exportin/karyopherin transport receptor family nor on directional signals from Ran. Rather, other distinct, conserved proteins mediate mRNA nuclear export, one of which shares with exportins/karyopherins the property of binding directly to FG repeat nucleoporins.

Bulk mRNA export is independent of CRM1 and Ran

Because of the diversity of its substrates, CRM1 seemed a logical candidate for mediating cellular mRNA export, an expectation supported by one study of a ts yeast Crm1p mutant [150]. However, in several other studies, inhibition of CRM1's activity either failed to affect mRNA export or affected export only after prolonged CRM1 inactivation, suggesting that export defects were indirect manifestations of CRM1 inhibition [151–153]. In fact, CRM1 inactivation leads to nuclear accumulation of normally cytoplasmic RanGAP and RanBP1 (see review by [154]), which would be expected to impair reimport of factors necessary for mRNA export. Similarly, genetic and oocyte injection experiments suggested that the Ran system is required for mRNA export [33, 155]. However, the apparent requirement for RanGTP in mRNA export may have reflected the need to recycle non-importin β-like export factors (see below), a conjecture supported by more recent experiments [156]. Thus, although there are reports implicating exportins/karyopherins in export of some mRNAs (discussed later), current attention has focused on other factors involved in mRNA export.

The TAP/Mex67p pathway
A new window into mRNA export was opened with identification of the essential yeast protein Mex67p and its metazoan orthologue TAP [157–159]. Temperature-sensitive Mex67p mutants rapidly accumulate poly(A) RNA in the nucleus, ultraviolet (UV) crosslinking shows that Mex67p is bound to poly(A) RNA and GFP-tagged Mex67p is found primarily associated with NPCs. TAP, which can functionally replace Mex67p in yeast [78], was initially identified as a cellular factor responsible for export of intron-containing D-type retroviral mRNAs, by binding the cis-acting constitutive transport element (CTE) within these mRNAs. A role for TAP in cellular mRNA export was first revealed in oocyte injection experiments, in which CTE RNA competed in a TAP-dependent manner with mRNA export [158, 160]. TAP is also essential for mRNA export in *C. elegans* and *Drosophila* [161–163] and stimulates mRNA export in somatic mammalian cells [164, 165].
TAP and Mex67p shuttle between the nucleus and cytoplasm, consistent with a role in mRNA export [78, 159, 166-168]. They are members of a family of related, evolutionarily conserved proteins, the NXF family [79] (fig. 4). These proteins bind directly to FG repeat nucleoporins located in the channel of the NPC. NPC binding occurs through a C-terminal UBA (ubiquitin-associated fold) domain and a domain immediately preceding the UBA domain that resembles the import receptor NTF2. In addition, TAP and Mex67p form heterodimers with small proteins, the NTF2-like protein p15 (also known as NXT1) and Mtr2p, respectively [78, 166, 169]. TAP and p15 interact with each other through their shared NTF2 folds, thereby resembling the homodimeric import receptor NTF2. It has been suggested that p15 serves to bring about the proper folding of the NTF2-like domain within TAP, which in turn binds FG repeat nucleoporins of the NPC.
Mtr2p and p15 do not show sequence similarity, but co-expression of p15 with TAP is required for TAP to functionally replace Mex67p in yeast, suggesting that Mtr2p and p15 are functionally equivalent. Further, like Mtr2p, metazoan p15 is also essential for mRNA export [162, 170]. In yeast, Mtr2p is required for Mex67p to associate with the NPC [166]. Interestingly, in metazoans, export of cellular mRNA requires that TAP have two nucleoporin binding sites. However, export of CTE-containing RNAs can be mediated by TAP having only a single nucleoporin binding domain, either the NTF2-like domain or the UBA domain, and thus CTE-RNA export can be independent of p15 [171].

How do TAP/p15 and Mex67p/Mtr2p find their mRNA ligands?
TAP behaves as a sequence-specific RNA binding protein since it binds directly to a wild-type CTE but not to slight variant sequences [158, 172]. However, CTE elements are not apparent in bulk cellular mRNA. Rather, TAP binds cellular mRNA by protein-protein interaction. In yeast, the bridging factor is Yra1p [173, 174], a member of a conserved family of RNA-binding proteins called REFs [174]. Murine REF1, also known as Aly, is interchangeable with Yra1p, both biochemically and genetically. In oocyte injection experiments, recombinant Aly stimulated mRNA export and anti-REF antibodies inhibited export [175], further direct evidence that REFs are involved in mRNA export. Importantly, the antibodies did not block nuclear export of CTE-containing RNAs, which is mediated directly by TAP, indicating that REFs function at a step prior to involvement of TAP/Mex67p.

Splicing-mediated recruitment of REFs to mRNAs

One step prior to TAP/Mex67p in which REFs are encountered is pre-mRNA splicing, since splicing recruits REFs to mRNA. In oocyte injection experiments, mRNAs generated by splicing were exported more rapidly than identical complementary RNA (cDNA) transcripts [176]. The spliced mRNA and cDNA transcripts assembled into different RNPs, and assembly into splicing-derived RNPs resulted in faster export [176, 177]. Further, Aly was associated with the splicing-derived mRNP but not its unspliced counterpart [178, 179]. Subsequent studies revealed that splicing places on the mRNA, ~20–24 nucleotides upstream of exon-exon junctions, a tightly bound complex of proteins dubbed the EJC (exon junction complex), one component of which is

Figure 4. Domain organization of TAP family of nuclear export factors. TAP proteins, also known as NXFs, contain a less-conserved N-terminal domain (purple) varying in size among different members of the family and within which is a nuclear localization signal (NLS). Following the N-terminal domain is an RNA binding domain (RBD) found only in human, *Drosophila* and *C. elegans* NXFs. LRR is a leucine-rich repeat domain, followed by the highly conserved NTF2-like domain, which allows heterodimerization with p15 (NXT1). p15 is also related to NTF2, and the structure formed between p15 and the TAP NTF2-like domain has an overall structure similar to the NTF2 homodimer. The C-terminus of the TAP family members contains a ubiquitin-associated fold (UBA) domain. Both the NTF2-like-p15 complex and the UBA domain are responsible for NPC interaction. Figure adapted from [79] with permission. © Urban & Fischer Verlag.

Aly [179-181]. Since Aly functions in mRNA export and binds TAP, the splicing-dependent deposition of Aly onto mRNA nicely explained the more efficient export of spliced transcripts and suggested that splicing-dependent alteration of the mRNP is a mechanism that both enhances interaction with subsequent export steps as well as allows for preferential export of fully spliced mRNAs.

While Aly is an adaptor that recruits TAP, Aly itself is recruited to mRNA by another factor, UAP56 [182]. UAP56 is a conserved DEAD-box helicase that acts at an early step in spliceosome assembly [183]. UAP56 binds Aly directly, with high affinity, and independent of RNA. UAP56 is present in equimolar amount with Aly in spliced-derived mRNPs, and Aly mutants unable to bind UAP56 fail to be recruited to the spliced mRNP. Just when and how the other members of the EJC are recruited remains to be determined.

Splicing-mediated recruitment of mRNA export factors (see fig. 5) is an attractive mechanism, since the vast majority of metazoan mRNAs are spliced and since such a mechanism could provide, in addition to nuclear retention, another means to prevent export of unspliced transcripts. However, RNA interference (RNAi)-mediated depletion of any, or all *Drosophila* EJC components has revealed that the EJC is dispensable for mRNA export [184]. Some nuclear accumulation of poly(A) was observed in EJC-depleted cells, but it was substantially less than that seen when either TAP or UAP56 was depleted. As Gatfield and Izaurralde suggest, aside from REFs, another essential or partially redundant adaptor(s) may link TAP to UAP56. Furthermore, UAP56 is found along the mRNA independent of splicing [185]. Thus, UAP56 does not necessarily function only to recruit Aly, and it may perform some other, essential function in mRNA export. Indeed, UAP56 is also required for export of mRNAs that are not derived by splicing [186].

Cotranscriptional recruitment of export factors to mRNA

Unlike metazoans, solid evidence for a splicing-dependent complex comparable to the EJC has not been obtained for yeast (but see [187]). In any case, while almost all metazoan mRNAs are derived from splicing, only 5% of yeast genes have introns. As such, cotranscriptional recruitment appears to be one mechanism for association of export factors with yeast mRNA. Mutations in Sub2p, a factor homologous to UAP56 and also implicated in splicing [188–190], cause mRNA export defects, but this effect is likely unrelated to defective splicing, since export of mRNAs derived from intronless genes is also blocked [191, 192]. Rather, Sub2p and Yra1p are recruited to transcribing genes by way of THO, a complex involved in transcription elongation [193–196].

Yeast THO consists of five proteins, Tho2p, Hpr1p, Mft1p, Thp2p and Tex1p [195, 196]. Sub2p and Yra1p copurify with THO in an RNase insensitive manner and also interact genetically with all of the THO proteins [194, 195, 197]. This supercomplex has been called the TREX (transcription/export) complex. Deletion of any THO component causes a complete lack of interaction between the remaining subunits, as well as nuclear accumulation of mRNA. Sub2p binds directly to the Hpr1p subunit of THO, thereby bridging the interaction between THO and Yra1p [191, 194, 195]. Biochemical evidence suggests that a TREX complex exists in mammals as well [195].

The observation that a complex involved in transcription elongation interacts with mRNA export factors led to examination of the association of these factors with transcribing genes. In chromatin immunoprecipitation (ChIP) experiments, components of TREX are indeed found associated with chromatin, and, importantly, mirroring the binding assays, the THO subunit Hpr1p was required for efficient association of Sub2p and Yra1p as assayed by ChiP [194]. Strikingly, the TREX complex is specifically recruited only to genes undergoing transcription [194, 195].

Because mutations in TREX components all cause nuclear accumulation of mRNA, the presumption is that the export defects are due to inadequate loading of export factors onto emerging mRNAs. Experiments to date do not indicate exactly how Sub2p and UAP56 become associated with nascent transcripts. For example, do these factors, with or without their partners Yra1p and Aly, bind first to the THO complex and then become transferred to the mRNA, or does THO in conjunction with the mRNA stimulate direct binding of Sub2p/UAP56 to the nascent transcript? The first alternative would seem the more likely possibility, since all of the proteins copurify in an RNA-independent manner. Also, a handing-off mechanism might work best for delivery of export factors onto mRNAs lacking introns, and as previously indicated, UAP56 is required for export of both intron-containing and some intronless mRNAs [186].

What about the 5% of yeast mRNAs derived from intron-containing genes? Does THO contribute to export factor loading onto these transcripts? Since Sub2p/UAP56 are implicated in spliceosome assembly, they need not necessarily make their way onto mRNA via RNA Pol II/THO, but they could arrive as the spliceosome assembles. Indeed, for the yeast *DBP2* gene, Yra1p association appears to occur primarily as a consequence of splicing; mutants blocked early in spliceosome formation showed reduced association of Yra1p with *DBP2*, while mutants in which release of the mRNA from the spliceosome was slowed exhibited increased association (presumably because there was more time for Yra1p to find the transcript) [187]. If the traveling polymerase complex had been

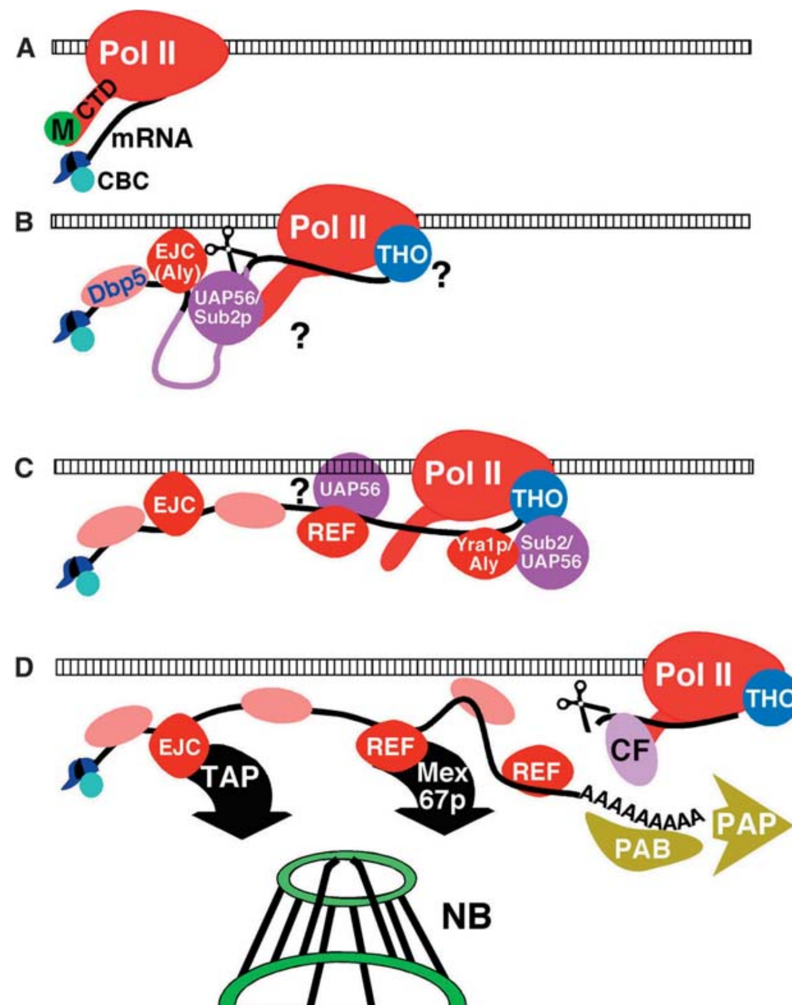

Figure 5. mRNA export. (*A*) After the initial 20–30 nucleotides of an mRNA have been polymerized, the 7-methyl-G cap structure is formed on the 5′ end of the transcript by a set of enzymes (**M**) recruited by the carboxyl-terminal domain (**CTD**) of the large subunit of RNA polymerase II (**Pol II**; see [144]). The cap structure is then recognized by a heterodimer, the cap-binding complex (**CBC**), which accompanies the mRNA through the NPC and is replaced in the cytoplasm by translation initiation factors [307]; the cap-CBC complex probably adds to the efficiency of mRNA export by facilitating exit of mRNAs through the NPC beginning with their 5′ ends, but cap-CBC is not absolutely required for export [308]. The DEAD box RNA helicase **Dbp5** binds very early during transcription and is ultimately found distributed along the entire mRNA transcript [217]. (*B*) In metazoans, in which essentially all mRNAs contain introns, a complex of five proteins, the **EJC**, is deposited on mRNAs as a consequence of splicing, ~20–24 residues 5′ of the resulting exon-exon junctions. Among the EJC constituents is **Aly**, a member of the REF family of export adaptors. Aly, and perhaps the entire EJC, is recruited to the RNA by **UAP56/Sub2p**. Although UAP56/Sub2p are implicated in splicing, their precise function in that regard is unknown; the figure is meant to show UAP56/Sub2p associated during the splicing process (**scissors**), but whether it is part of the spliceosome and/or bound to the intron or an exon is not known (hence the question mark). Indeed, UAP56/Sub2p is also found along the mRNA independent of splicing (see C). (*C*) In metazoans, an unstructured segment is sufficient to 'identify' a transcript as an mRNA and lead to association of the mRNA with REF [210]. Since UAP56 is responsible for recruiting REFs by splicing and transcriptional mechanisms, and it is found all along an mRNA, UAP56 may also be involved in recruiting REF in the context of an unstructured RNA element, although at present UAP56's participation in this process has not been established (hence the?). In *S. cerevisiae*, only 5% of genes contain introns, so that association of export factors with mRNAs occurs, in part, by co-transcriptional mechanisms. **THO**, a complex implicated in polymerase elongation, recruits Sub2p, which in turn recruits Yra1p (an Aly orthologue). Biochemical evidence suggests that a complex of similar composition to that of THO exists in animal cells and that metazoan THO binds UAP56. Note that one component of the yeast THO complex, Hpr1p, copurifies with one particular form of Pol II, suggesting that THO and Pol II associate with each other during transcription; however, there is as yet no evidence for a direct role for THO in transcription [196]. Thus, as indicated in **B**, just when THO becomes associated with the gene is uncertain, and the figure is not meant to imply that it is part of the Pol II complex. In fact, some THO components display RNA-binding activity so that THO may be partly or wholly associated with the mRNA [196]. (*D*) Proper 3′ end cleavage and polyadenylation also contribute to recruitment of mRNA export factors, as well as being necessary for release of finished transcripts from their sites of transcription. **CF** is a cleavage factor, mutations in which reduce association of Yra1p with nascent transcripts. Because of its association with RNA Pol II during transcription initiation and elongation, CF may recruit Yra1p cotranscriptionally (not shown). **TAP** and **Mex67p**, in conjunction with their small partners p15 and Mtr2p (not shown), are the actual transport factors, binding Aly and Yra1p, respectively, and making contact with nucleoporins in the NPC nuclear basket and with FG-repeat nucleoporins in the NPC channel. **PAB** and **PAP** are the poly(A)-binding protein and poly(A) polymerase.

competent to deliver export factors, defects in splicing should not have seriously affected delivery of Yra1p to the *DBP2* pre-mRNA. Curiously, the intron in *DBP2* is rather uncharacteristically positioned, being more than 1 kb downstream of the 5′ end, a distance that one might have expected sufficient for Pol II/THO to deliver Yra1p to the early splicing defective transcript, even before the spliceosome had assembled. Notably, the 5′ splice junctions in most intron-containing yeast mRNAs are very close to the 5′ end of the transcripts. It remains to be investigated whether splicing plays a prominent role in delivery of the factors to these splicing-derived yeast mRNAs .

Another conundrum is the fact that yeast THO is not essential for viability and is present in substoichiometic amounts compared with Pol II. How then can, or does, THO provide for export factor loading on the ~95% of yeast mRNAs lacking introns? Perhaps in most cases the factors bind directly to the mRNA by way of some more general property of all such transcripts (see below), while THO may operate at only a subset of genes, i.e. those genes needing assistance from a processivity factor. Interestingly, Yra1p was totally absent from the 35S ribosomal DNA gene when that gene was transcribed by a Pol II promoter [187], suggesting that Pol II alone is insufficient to recruit export factors to the average (intronless) yeast mRNA. But this result may be difficult to interpret, since the 35S ribosomal RNA (rRNA) is presumably highly structured and subject to a number of processing steps whose trans-acting complexes may have interfered with Yra1p association. In another study, T7 RNA polymerase was used to transcribe an intronless GFP sequence in yeast, and the resulting mRNA was exported to the cytoplasm [198]. Thus, neither transcription by Pol II nor splicing is necessary for export, although the relative export efficiency of T7 and Pol II-derived transcripts was not assessed. Notably, export of the T7-GFP transcript did require proper 3′ processing, a step that also appears to recruit Yra1p (see next).

Coupling of mRNA export/recruitment of export factors to 3′ end formation

The 3′ ends of metazoan mRNAs are formed by endonucleolytic cleavage at a site located between a conserved upstream AAUAAA sequence and a less conserved downstream U- or GU-rich motif; cleavage is performed by a supramolecular assembly of at least three multiprotein complexes. The cleavage complex also recruits the poly(A) polymerase which, together with a specific poly(A)-binding protein, catalyzes addition of ~200 adenines onto the newly created 3′ end. Signals for 3′ processing in yeast differ from metazoan signals in both sequence and arrangement, and are less conserved; nonetheless, the cleavage and polyadenylation protein complexes are generally conserved in eukaryotes [199].

Efficient export of mRNA has long been suggested to depend on correct cleavage and polyadenylation. Transcripts whose 3′ ends are produced by artificial means bypassing the normal cleavage/polyadenylation process are exported poorly, and inserting a long poly(A) stretch upstream of unnaturally formed mRNA termini is insufficient to restore export [198, 200–202] (but see [203] for an exception). Inactivation of the poly(A) polymerase or CFI (involved in 3′ cleavage) also causes nuclear retention of mRNAs [204]. Thus, both cleavage and polyadenylation, or the cleavage/polyadenylation factors themselves, are required for efficient mRNA export. The molecular mechanism(s) whereby improperly cleaved or polyadenylated mRNA becomes export incompetent is unclear, but two phenomena appear to be at work, active retention of aberrant transcripts and lack of export factor recruitment to improperly processed transcripts.

In yeast, a variety of mutants defective in general mRNA export accumulated mRNAs at their sites of transcription [191, 192, 197, 202, 205, 206]. Also, ts mutations in poly(A) polymerase or CFI, or cells in which mRNA ends were generated by a self-cleaving ribozyme, accumulated mRNAs at transcription sites. In all cases, mRNA sequestration required Rrp6p [197, 207], one of a number of exoribonucleases that comprise the nuclear exosome, whose functions include RNA degradation [208]. Thus, mRNAs with abnormal 3′ ends or which are prevented from being exported are retained at their sites of transcription, and the nuclear exosome appears to participate in a mechanism that assesses correct 3′ end formation. How the transcripts are retained is unknown; furthermore, while Rrp6p is clearly involved in detecting the 3′-defective transcripts, detection can occur independently of a polyadenylation signal and its associated factors, since a self-cleaved transcript was also retained. Related mechanisms may operate in metazoans; a β-globin transgene unable to undergo correct 3′ end formation produced an RNA that was not cleaved and remained in close proximity to its site of transcription [209].

As mentioned, the presence of a poly(A) tail in a transcript with an artificially generated 3′ end is insufficient for export; mRNAs must be actively processed by the normal machinery in order for entry into the export pathway. Consistent with this idea, some ts alleles of CFI produced transcripts that were polyadenylated by an apparently aberrant mechanism, at sites downstream from the normal cleavage site, and these distally adenylated transcripts were not exported [202]. Likewise, deletion of the CFI processing site in an mRNA produced elongated, polyadenylated, export incompetent transcripts. These findings suggest that the 3′-processing apparatus recruits export factors, and indeed, a substantial decrease in Yra1p association with both intron-containing and intronless genes was observed by ChIP in yeast strains with

ts CFI mutations [187]. Still, why should lack of 3′ cleavage result in the near absence of Yra1p when other mechanisms can also recruit Yra1p? Cleavage and polyadenylation factors associate with RNA Pol II during formation of the transcription initiation complex [144, 145]. Perhaps, in a manner akin to THO, as 3′-processing factors travel with the polymerase they recruit export factors to the growing mRNA; the effect of a ts mutation in CFI may have been either a defect in its preinitiation association with RNA Pol II or in its subsequent export factor recruitment. These possibilities await biochemical analysis.

Recruitment of export factors by mRNA 'identity' elements

Transcription, splicing and 3′ end formation all influence mRNA export, contributing to its specificity and efficiency by recruiting export factors. However, none of these processes is absolutely required for mRNA export. Indeed, in frog oocytes, mature mRNAs that are not derived by transcription or splicing, and which are not 3′ cleaved and polyadenylated, are nonetheless exported, albeit less efficiently than transcripts produced by more natural means. Second, 95% of yeast genes and ~5% of metazoan genes lack introns, but their mRNAs are also efficiently exported. Likewise, synthesis by Pol II is not a guarantee that a transcript will enter the Mex67p/TAP export pathway, since U snRNAs are also transcribed by RNA Pol II and 5′ capped, but they are exported by CRM1.

Thus, what else makes an mRNA exportable, and what distinguishes a Pol II U snRNA transcript from a Pol II mRNA transcript? Recent work by Ohno et al. demonstrates that an unstructured segment of RNA contributes to mRNA identity and export and that splicing not only steers a Pol II transcript into the TAP export pathway but blocks access to the U snRNA export pathway [210, 211]. For example, insertion of an intron into the normally intronless U1 snRNA caused the RNA to be spliced and exported in a CRM1-independent (LMB-insensitive) manner. As well, export of spliced U1 snRNA was competed by excess CTE and unaffected by a reduction in nuclear RanGTP concentration, both hallmarks of the TAP-mediated mRNA export pathway. Importantly, spliced U1 snRNA could not enter the U1 export pathway even when the mRNA export pathway was blocked by excess CTE, suggesting that the process of splicing or mRNA export factors prevented the spliced U1 RNA from interacting with U snRNA export factors. Indeed, spliced U1 snRNA could not be immunoprecipitated with either anti-CRM1 or anti-PHAX antibodies, even though the binding site for these export factors (the m^7G cap) was present in the RNA (PHAX bridges the interaction of CRM1 with CBC bound to the U1 snRNA cap). However, antibodies to components of the EJC effectively precipitated the spliced snRNA. Thus, splicing 'redefined' U1 as an mRNA.

Some mRNAs are naturally intronless, and in fact, intronless mRNA injected into oocytes associates (albeit inefficiently) with EJC components (when capped, intronless mRNA nonetheless does not associate with PHAX) [178, 180, 210]. Interestingly, when splicing was inhibited, U1 snRNA containing an intron associated with Y14 (an EJC component) but not with PHAX, prompting Ohno et al. to ask whether insertion of any sequence would cause U1 to behave as an mRNA. Indeed, insertion of any of several exonic sequences, in either orientation, caused U1 to be exported in a CRM1/PHAX-independent, TAP-dependent manner. Thus, both the presence of an intron as well as more ill-defined aspects of an mRNA, such as an extended unstructured region, serve as mRNA 'identity elements'. Surprisingly, the presence of an intron in U1 even in the absence of splicing was sufficient to preclude association with snRNA export factors, suggesting that introns may serve two purposes, to be recognized as 'mRNA-like' rather than as an intron per se, thereby precommitting the transcript to the mRNA export pathway even before spliceosome assembly begins, and then to cause placement of the EJC on the RNA following splicing. It will be interesting to determine just how refashioning the RNP for export via the mRNA pathway either displaces or prevents association of PHAX and CRM1, which essentially bind to the extreme 5′ end of U1 snRNA.

Beyond Mex67p/TAP

Mex67p/TAP connect export with transcription and RNA processing and they bind directly to the FG-repeat nucleoporins to mediate the translocation stage of export. Sandwiched between these two phases appears to be a step in which the outgoing mRNP docks at the nuclear basket before entering the NPC channel. In yeast, docking has been suggested to occur by way of a complex of two factors, Sac3p and Thp1p, which binds Mex67p and Nup1p, a constituent of the basket [212].

How is the docked mRNP released and compelled to travel through the NPC, in the correct direction? In addition to factors already described, many other proteins, e.g. the cap-binding complex, hnRNP proteins and assorted splicing factors, associate with mRNAs during transcription, splicing and export. Some factors are removed during NPC transit, while others dissociate only after entering the cytoplasm or when the mRNA begins translation [54]. Thus, an mRNP undergoes continual and substantial remodeling during its lifetime. These 'changes in state' may help advance the mRNP along its journey through the NPC. Intriguingly, a putative RNA helicase and RNA-dependent ATPase, Dbp5p, is required for mRNA export in yeast and animal cells [213–215]. RNA helicases can disrupt both RNA-RNA and RNA-protein interactions [216]. Dbp5 is primarily cytoplasmic, associating with CAN/Nup159 within the fibrils extending from the NPC

into the cytoplasm; thus Dbp5 is positioned to function in late steps of mRNA export, promoting conformational changes in the mRNP necessary for movement into the cytoplasm. Again, this restructuring could confer directionality to mRNA export, especially if one function of Dbp5 is to discharge export factors from the mRNP. Actually, in *Chironomus tentans*, Dbp5 binds cotranscriptionally to pre-mRNA, accompanying it to, and through, the NPC and into the cytoplasm [217]. Thus, Dbp5's role in mRNP biogenesis appears to begin at the transcriptional step, and it may be responsible for remodeling of the mRNP – unfolding, repacking and exchanging proteins – during all of an mRNAs nuclear undertakings, i.e. transcription, processing and export.

Wither exportins/karyopherins in mRNA export?
Although CRM1 is not responsible for bulk mRNA export, specific cellular mRNAs and certain viral mRNAs (see [115] and references therein) make use of the CRM1 export pathway. Most compelling is the case of NXF3, a TAP isoform, that can function as an mRNA export factor and which is found associated with poly(A) RNA [218]. Conspicuously, NXF3 lacks the C-terminal domain of TAP that binds FG nucleoporins. Instead, NXF3 contains a functional Rev-like CRM1 interaction domain, located in what would be the CTE-binding domain of TAP. NXF3 is found primarily in testis with traces found in other tissues (TAP is ubiquitous and highly abundant). Thus, while NXF3's mRNA targets are unknown, NXF3 may act as a tissue-specific mRNA export factor that, like some viral mRNAs, accesses the CRM1 pathway.
Nuclear export of the c-*fos* mRNA is another case in which importin β-like factors have been implicated. Like many short-lived cytokine and protooncogene mRNAs, c-*fos* mRNA contains an A+U-rich 3′ UTR element (ARE) that regulates its half-life through interaction with several proteins, including HuR. HuR has been reported to mediate nuclear export of c-*fos* by interacting with either CRM1 or a previously identified importin, transportin 2 [219]. Interestingly, transportin 2 can also interact directly with TAP, and transportin 2 has been suggested to serve as an mRNA export receptor [220]. Interference with transportin 2 function either by RNAi or by competition with competing, cell-permeable peptides resulted in nuclear accumulation of poly(A) RNA. However, the interaction between transportin 2 and HuR or TAP is highly sensitive to RanGTP, indicative of an involvement of transportin 2 in the nuclear import of the two factors. Indeed, HuR and TAP are imported into the nucleus by transportin 1 and 2 with comparable efficiencies [S. Güttinger and U. Kutay, unpublished]. Therefore, the observed mRNA export defect attributed to interruption of transportin 2 activity may be indirect and due to a deficiency in nuclear import of mRNA binding and export proteins.
Finally, as mentioned above, certain viral mRNAS are exported by use of *cis*-acting sequences that bind either virally-encoded or cellular RNA-binding proteins. The viral proteins (e.g. Rev) serve as adaptors that access the CRM1 export pathway, while export of viral mRNAs mediated by cellular factors has been found to be independent of CRM1. Recently, however, a cis-acting element in woodchuck hepatitis virus (WHV) mRNA, which requires no viral proteins for function, has been found to access both a CRM1-dependent and a CRM1-independent export pathway [115]. Interestingly, export of c-*fos* mRNA was also reported to be partially dependent on CRM1 and partly independent of CRM1 [219]. It remains to be seen whether c-*fos* and WHV are rare exceptions or whether other mRNAs rely on export mechanisms that do not involve TAP/Mex67p.

Export of ribosomal subunits
Like mRNA, ribosomes constitute another very complex cargo that must be exported out of the nucleus. Ribosomal subunits are assembled in the nucleolus from rRNA and ⊕80 distinct ribosomal proteins. A large pre-rRNA is transcribed by RNA Pol I, and this precursor is processed into 18S, 5.8S and 25S RNA. 18S rRNA is present in the small 40S subunit, while the larger 60S subunit contains the 25S and 5.8S rRNAs as well as a 5S rRNA synthesized separately by RNA Pol III. Ribosomal biogenesis requires a multitude of trans-acting factors such as RNases, RNA-modifying enzymes, RNA helicases, AAA-type ATPases (ATPases associated with various cellular activities), GTPases and others (for review see [221]). Their actions ensure a proper coordination of rRNA processing and modification, ribosomal subunit assembly and, finally, nuclear export (reviewed by [222–224]).
Over the past couple of years many trans-acting factors have been identified that assist in the final steps of ribosomal assembly and in nuclear export, using mainly genetic screens and proteomic approaches in yeast. The multitude of factors identified so far has coincided with the preconception that ribosome biogenesis is a highly complex affair, since the specific functions of most of the newly identified proteins remain to be determined in molecular detail. Many of the identified trans-acting factors localize to the nucleolus, and perturbing their functions results in defects in ribosomal processing and assembly. Proteins relevant to nuclear export of ribosomes might be those that are associated with export-competent subunits in the nucleoplasm and which, if mutated, lead to accumulation of premature ribosomal particles containing correctly processed forms of nuclear rRNAs. Alternatively, similar to what has been observed for mRNA, export adaptors may be deposited early on nascent ribosomes, and defects in their respective genes may interfere with export earlier, during ribosomal assembly.

Initial injection experiments in *Xenopus* oocytes suggested that ribosomal subunit export is an energy-dependent, receptor-mediated process [225]. These early studies also indicated that ribosomal subunits can be exported independent of each other. This finding has been supported by numerous genetic experiments in yeast. Mutations in factors specifically required for the assembly of one subunit selectively interfere with the biogenesis of the one but not the other subunit. The joining of ribosomal subunits in the nucleus is likely prevented by Tif6p, a nuclear protein stably associated with pre-60S subunits [226, 227]. Tif6p removal from 60S subunits occurs in the cytoplasm and is promoted by the cytoplasmic GTPase Efl1p [227, 228].

In yeast, the favored candidate for a ribosomal subunit nuclear export factor is Crm1p. Export of both 40S and 60S subunits has been shown to depend on functional Crm1p and an intact RanGTPase system [229–232]. CRM1 also is involved in export of ribosomal subunits in animal cells (see below). However, interaction of CRM1 with either of the two subunits, either direct or indirect, has yet to be demonstrated in any system.

60 S subunit export

Hurt et al. developed a yeast in vivo nuclear export assay for the large ribosomal subunit based on green fluorescent protein (GFP)-tagged ribosomal protein L25 (Rpl25p-GFP) [233]. Mutations in genes encoding components of the RanGTPase system and certain nucleoporins interfered with 60S subunit biogenesis and led to nuclear accumulation of Rpl25p-GFP. A similar approach taken by Silver et al. using a GFP fusion of Rpl11p, a protein suggested to assemble late into nascent subunits, gave essentially the same results [234]. Subsequently, a genetic screen for **ri**bosomal e**x**port mutants (rix mutants) [229], genetic interaction screens, and mass-spectrometric analyses of preribosomal particles added new factors to the inventory. In all, about 100 trans-acting factors are believed to be involved in ribosomal biogenesis and/or export.

Many of these trans-acting factors, including proteins and RNPs responsible for rRNA processing and modification, are required early in the assembly pathway, and interference with their functions leads to a nucleolar accumulation of ribosomal subunit precursors. In addition, some factors might be required for release of the subunit from the nucleolus, which may require either structural changes in the pre-60S itself, gain of a different set of assembly factors or dissociation from nucleolar retention sites. ATPases and GTPases are good candidates for participating in this step. The AAA family member Rix7p has been shown to be required for the release of pre-60S from the nucleolus and subsequent transport to the nuclear periphery [235]. Rix7p appears to associate with the preribosomal subunit only transiently. It is unknown at which precise step the potential ATPase activity of Rix7p is required. Another factor acting roughly at the same stage of preribosomal assembly is Noc1p [236]. Noc1p forms a complex with Noc2p that unlike Noc1p, is also found in the nucleoplasm. There, Noc2p is instead associated with the Noc1p-related factor Noc3p. While the Noc1p/Noc2p complex is associated with early 90S complex as well as 60S preribosomes in the nucleolus, the Noc2p/Noc3p is bound to 60S preribosomes in the nucleoplasm [236]. Apparently, release from the nucleolus is accompanied by a change in preribosomal subunit composition. This is also reflected in the behavior of Rlp7p and Rlp24p, two nuclear proteins related to ribosomal protein Rpl7p and Rpl24p, respectively, that associate with the pre-60S particles only transiently [237–239]. Affinity purification of distinct nucleolar and nucleoplasmic pre-60S particles followed by determination of their respective protein composition confirmed that nucleoplasmic pre-60S particles have lost a number of nucleolar assembly factors and gained a different set of trans-acting factors [240]. Interestingly, a couple of putative GTPases such as Nog1p, Nug2p/Nog2p and Nug1p are associated with nucleolar and nucleoplasmic preribosomal particles [237, 241, 242]. The GTPases may help coordinate, monitor or induce changes in preribosomal subunit composition along the assembly pathway.

Interference with the function of a second group of factors leads to a nucleoplasmic accumulation of preribosomal subunits. Among these factors might be components needed for the export step per se. Two rix mutants, rix1 and rix5, cause a predominantly nucleoplasmic accumulation of pre-60S [229]. RIX5 codes for ribosomal protein L10 (Rpl10p) needed for joining of 60S with 40S subunits in the cytoplasm [243]. Rpl10p interacts with Nmd3p, a nonribosomal protein associated with 60S subunits that has been proposed to serve as an export adaptor [229, 230]. Nmd3p is conserved from archaebacteria to humans and might possess a primary function in ribosomal biogenesis unrelated to nuclear export. However, Nmd3p in eukaryotes is a shuttling protein that has acquired an additional C-terminal domain containing a leucine-rich NES that is required for cytoplasmic localization of Nmd3p [229, 230]. Defects in export of 60S subunits have been observed in mutants harboring NMD3 ts alleles. Moreover, overexpression of an NES deletion mutant of Nmd3p exerts a dominant-negative inhibition on 60S export. Based on these data, it has been suggested that Rpl10p serves as a landing pad for Nmd3p on nucleoplasmic pre-60S subunits [229]. Since nuclear export of 60S subunits also depends on functional Crm1p, the most straightforward model which condenses all of the data is that Nmd3p, by virtue of its C-terminal NES, recruits Crm1p to pre-60S subunits, which in turn mediates nuclear pore passage of this particle [229, 230]. However, so

far, it has not been demonstrated that Nmd3p binds to Crm1p, nor has Crm1p or Ran been detected on any of the analyzed 60S or pre-60S particles. The functions of CRM1 and NMD3 appear to be conserved from yeast to higher eukaryotes. Inhibition of the CRM1 export pathway leads to a defect in nuclear export of 60S subunits in HeLa cells and *Xenopus* oocytes [309, 310]. Like yeast Nmd3p, human NMD3 is a shuttling protein. CRM1 can bind directly to the conserved, C-terminal NES of human NMD3 [310]. Expression of mutant forms of NMD3 lacking the NES causes a dominant-negative inhibition of 60S subunit export in *Xenopus* oocytes [309]. These data from higher eukaryotes support the idea of a direct function of CRM1 and NMD3 in 60S subunit export.

Another candidate export mediator for pre-60S is yeast Mtr2p, the small subunit of the heterodimeric Mex67p/Mtr2p mRNA export factor. The function of Mtr2p in mRNA export can be separated from its potential function in 60S subunit export, since specific *MTR2* alleles affect one pathway but not the other [234, 241]. Moreover, the *MEX67* alleles tested so far have no defect in 60S export but are defective in mRNA export. Mtr2p can indeed be coisolated on late pre-60S biogenesis intermediates [240]. It will be interesting to see whether the function of Mtr2p in ribosomal export is indeed independent of Mex67p and how Mtr2p is recruited to 60S preribosomes.

40S subunit export

An elegant export assay monitoring nuclear accumulation of 40S preribosomal subunits in *S. cerevisiae* relies on the detection of the internally transcribed spacer 1 (ITS1) present in 20S pre-rRNA by FISH [231]. In yeast, ITS1 is processed in the cytoplasm after export of pre-40S subunit to yield mature 18S rRNA and is then degraded by the exoribonuclease Xrn1p. A nucleoplasmic accumulation of ITS1 could not only be observed in strains with mutations in certain nucleoporins but also in strains defective in the RanGTPase system, suggesting a role for these factors in pre-40S export [231, 232]. There are several indications that Crm1p is involved in the nuclear export of 40S subunits. First, a yeast strain having an LMB-sensitive form of Crm1p rapidly accumulates pre-40S particles in the nucleoplasm upon LMB treatment. Second, deletion of the Crm1p cofactor Yrb2p results in defective small subunit export [232]. Also in higher eukaryotes, a functional CRM1 export pathway is required for 40S biogenesis and export [309, 310]. It remains to be seen, however, whether Crm1p contributes directly to 40S export. This question awaits determination of whether Crm1p binds 40S subunits, either directly or by way of adaptor factors.

Candidate adaptor proteins might be among the nonribosomal proteins present in a late pre-40S assembly intermediate isolated by tandem affinity purification with tagged Enp1p [244]. Enp1p is found in a complex containing 20S pre-rRNA, an almost complete set of small ribosomal subunit proteins and a number of other factors. Since Enp1p is located primarily in the nucleus but can also be found in the cytoplasm, it may be part of an export competent complex. It is presently unclear what role Enp1p and other nonribosomal factors present in the same complex play in nuclear pore passage of the small subunit.

Interestingly, similar to the maturation of 60S particles, potential GTPases and Noc proteins play a role in 40S biogenesis. The GTP-binding protein Bms1p and Noc4p are required for early steps of rRNA processing [245–247]. In contrast, depletion of the nucleolar factor Tsr1p, related to Bms1p by sequence, results in accumulation of 20S pre-rRNA, suggesting that Tsr1p function could be required for the gain of export competency of pre-40S subunits [246].

Finally, aside from the identities and functions of factors that mediate ribosomal subunit assembly and nuclear export, other questions remain. In view of the nearly 2:1 mass ratio of RNA to protein in the ribosome, are there additional factors needed to shield the charged ribosomal surface from the hydrophobic NPC interior, in much the same way that hnRNP proteins are anticipated to help chaperone mRNPs? Also, because of their large sizes, do ribosomal subunits undergo specific conformational changes as they pass through the NPCs, and if so, what factors produce these structural alterations? In a similar vein, might remodeling or interaction of the subunits with NTPases during pore passage contribute to the driving force for transport, as is believed to be the case for mRNP export? Answers to such questions may depend on purification and functional testing of preribosomal subunits present in the nucleus and readied for export.

Concluding remarks

This review has focused on the mechanisms of nucleocytoplasmic transport and on the description of nuclear transport pathways important for housekeeping functions of a eukaryotic cell. Another important aspect of nucleocytoplasmic transport is its regulation, the control of substrate transport in response to, e.g., environmental, cell cycle, apoptotic and developmental signals [248]. Regulation of transport occurs mainly through phosphorylation and dephosphorylation of cargo proteins, which controls access to the nuclear transport machinery or binding to immobile factors that retain substrates in a compartment. Other posttranslational modifications may also turn out to be equally important in regulated cargo transport. As well, additional examples of how nucleocytoplasmic transport contributes to the regulated communication in a eukaryotic cell likely will be revealed in future.

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