Mechanistic insights from structural analyses of Ran GTPase-driven nuclear export of proteins and RNAs

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Abstract

Understanding how macromolecules are rapidly exchanged between the nucleus and the cytoplasm through nuclear pore complexes (NPCs) is a fundamental problem in biology. Exportins are Ran GTPase-dependent nuclear transport factors that belong to the karyopherin-β family and mediate nuclear export of a plethora of proteins and RNAs, except for bulk mRNA nuclear export. Exportins bind cargo macromolecules in a Ran-GTP dependent manner in the nucleus, forming exportin-cargo-Ran-GTP complexes (nuclear export complexes). Transient weak interactions between exportins and nucleoporins containing characteristic phenylalanine-glycine (FG) repeat motifs facilitate NPC passage of nuclear export complexes. In the cytoplasm, nuclear export complexes are disassembled, thereby releasing the cargo. GTP hydrolysis by Ran promoted in the cytoplasm makes the disassembly reaction virtually irreversible and provides thermodynamic driving force for the overall export reaction. In the past decade, X-ray crystallography of some of the exportins in various functional states coupled with functional analyses, single particle electron microscopy, molecular dynamics simulations, and small-angle solution X-ray scattering has provided rich insights into the mechanism of cargo binding and release, and also beginning to elucidate how exportins interact with the FG repeat motifs. The knowledge gained from structural analyses of nuclear export is being translated into development of clinically useful inhibitors of nuclear export to treat human diseases such as cancer and influenza.

Keywords: exportin, Ran, nuclear export signal, nucleoporin, nuclear pore complex

Abbreviations used: NPC, nuclear pore complex; Nup, nucleoporin; NLS, nuclear localization signal; NES, nuclear export signal; MD, molecular dynamics; LMB, Leptomycin B; EM, electron microscopy; PDB, Protein Data Bank.

Introduction

In eukaryotic organisms, active transport of macromolecules between the nucleus and the cytoplasm is an essential cellular process. The nuclear transport occurs through nuclear pore complexes (NPCs) embedded in the nuclear envelope. The NPC is a huge supramolecular assembly constructed from multiple copies (8, 16, or 32) of approximately 30 different protein subunits (nucleoporins or Nups), with a total of approximately 500 Nups per NPC [1,2]. The NPC is equipped with a permeability barrier that prevents passive diffusion of inert objects > 5 nm in diameter [3] and yet facilitate active and selective transport of large macromolecules [4]. About one-third of Nups contain tandem amino acid sequence repeats, so-called FG (phenylalanine-glycine) repeats that have short hydrophobic cores containing phenylalanine and glycine residues with sequences such as FG, FxFG (where x is usually a small residue), or GLFG, separated by hydrophilic linkers of variable length and sequence. The FG repeat domains of Nups are natively unfolded [5] and are involved in forming the permeability barrier of NPCs [6-9].

The active transport pathways through NPCs are mediated by soluble nuclear transport receptors (NTRs). The NTRs circulate between the nucleus and the cytoplasm and transfer cargo macromolecules from one side of the nuclear envelope to the other [4]. All known NTRs can bind to FG-repeat containing Nups in such a way that allows the NTR-cargo complexes to penetrate the permeability barrier of NPCs [10,11]. The majority of NTRs are the karyopherin-β (Kap-β) family of proteins (14 in yeast and around 20 in metazoans) that include importins (nuclear import receptors), exportins (nuclear export receptors), and bidirectional Kap-βs that can mediate both nuclear import and export. Although the overall sequence similarity across Kap-βs is rather low (about 15-20%), all Kap-βs share a similar architecture composed of approximately 20

consecutive HEAT (for Huntingtin, Elongation factor 3, protein phosphatase 2A, and TOR kinase) repeats, each of which is made up of two antiparallel α-helices connected by a loop of varying length [12]. The HEAT repeats pack against each other, producing a gently curved super-helical structure with a continuous hydrophobic core, which is suitable to function as a coordinating scaffold to mediate protein-protein interactions [13]. Another functionally important feature of the HEAT repeat proteins is that they have conformational flexibility that can be utilized to regulate interactions with their substrates [14].

The nuclear transport pathways mediated by Kap-βs require the small GTPase Ran (Gsp1p in yeast), which is a highly conserved (80% identity among yeast and humans) member of the Ras superfamily of small GTPases. Ran represents a separate family among the Ras-related proteins. Ran is found predominantly in the nucleus, with a conspicuous acidic carboxy-terminal sequence devoid of an attachment signal for prenylation, which in other subfamilies are necessary for membrane targeting. Like other members of Ras superfamily, Ran acts as a molecular switch that cycles between GDP- and GTP-bound states [15]. The intrinsic rates of GTP hydrolysis and nucleotide release are slow, and the nucleotide bound state of Ran are modulated by at least two types of proteins: GEF (guanine nucleotide exchange factor) and GAP (GTPase activating protein). The chromatin-bound protein RCC1 (Prp20p in yeast), the GEF for Ran, is localized in the nucleus and increases the rate of release of bound GDP by ~10^5, allowing abundant intracellular GTP to bind Ran [16-18]. In contrast, RanGAP (Rna1p in yeast) is localized in the cytoplasm and increases the rate of GTP hydrolysis by ~10^5, returning Ran to the resting GDP-bound state [19-21]. The cytoplasmic Ran-binding proteins RanBP1 (Yrb1p in yeast) and RanBP2 function as co-activators of RanGAP [22]. The restrictions of the GEF to the nucleus and the GAP to the cytoplasm leads to the prediction that Ran exists primarily in the GTP-bound state in the nucleus, and

primarily in the GDP-bond state in the cytoplasm. This prediction of "Ran-GTP gradient" was verified experimentally by FRET (Fluorescence resonance energy transfer)-based imaging [23].

The Ran-GTP gradient is a crucial determinant of the directionality for both nuclear import and export mediated by Kap-βs [24,25]. In nuclear import, importin bind cargo in the cytoplasm and, after passing through NPCs, releases import cargo upon binding Ran-GTP in the nucleus [24-26]. The importin-Ran-GTP complex is recycled to the cytoplasm and disassembled by the action of RanBP1/2 and RanGAP [27-29], freeing importin for another import cycle and releasing Ran-GDP, which is then imported back to the nucleus by NTF2 (nuclear transport factor 2; the Ran-specific import receptor that does not belong to the Kap-β family) [30,31]. The GTP hydrolysis by Ran renders the disassembly reaction virtually irreversible. In contrast, export cargo and Ran-GTP bind cooperatively to exportin in the nucleus. After NPC passage, the nuclear export complex (exportin-cargo-Ran-GTP complex) is disassembled by the action of RanBP1/2 and RanGAP in the cytoplasm. The dissociated exportin and Ran-GDP are then recycled back to the nucleus to participate in another export cycle [32-36]. Thus both nuclear import and export pathways mediated by Kap-βs are coupled to GTP hydrolysis by Ran, which provides thermodynamic driving force for the overall transport reaction and allows accumulation of cargo against concentration gradient. It is important to note that the NPC passage step facilitated by transient binding of Kap-βs to the FG repeats is a reversible reaction [37], and does not impose directionality to nuclear transport. It is the disassembly of import or export complexes in the destination compartment that provides a Brownian ratchet mechanism to rectify transport of cargo in one direction.

Although structural study of exportins lagged behind that of importins, X-ray

crystallography in the last ten years of some (but not all) of the exportins in various functional states has provided rich insights into the molecular mechanism of nuclear export. This review will summarize the current state of understanding of the structural basis for Ran-dependent nuclear export pathways, especially in terms of the assembly and disassembly of the nuclear export complexes. For earlier review on this topic, the reader is referred to excellent reviews from the Chook [38,39], Conti [40,41], Tsukihara [42], Gorlich [43], and Ficner [44] groups.

Structures of Ran-GTP and Ran-GDP: active and inactive conformations

The structure of Ran was first determined in the GDP-bound conformation in 1995 [45]. The core of the globular domain of Ran (the G-domain) consists of a central β-sheet surrounded by α-helices, which is similar to other Ras-family GTPases such as Ras, Rab, and Arf [15]. In addition, Ran has a unique C-terminal extension that packs against the G-domain and ends in a α-helix in the GDP-bound Ran. The structure of GTP-bound Ran was obtained in 1999 from X-ray crystallography of the ternary complex formed between Ran, the slowly hydrolysable GTP analogue GppNHp, and effectors that specifically bind Ran-GTP with high affinity [46-48]. RanBP2 has four Ran-binding domains (RanBDs), each of which is highly homologous to RanBD of RanBP1 (Yrb1p), and the crystal structure of Ran-GppNHp was solved as a complex with the first RanBD (RanBD1) of RanBP2 [46]. Ran-GppNHp was also crystallized as a complex with the N-terminal fragment of human importin-β (also known as karyopherin-β1) [47], and as a complex with full-length human transportin (also known as karyopherin-β2) [48]. These structures showed that the major conformational changes of Ran between the GDP- and GTP-bound states are found in the switch I and switch II regions and the C-terminal extension (Fig. 1). Both switch regions I and II are involved in binding the γ-phosphate of GTP, and are relaxed into different

conformations after GTP hydrolysis and phosphate release. The conformational changes of the switch regions are coupled with drastic conformational rearrangements in the C-terminal extension of Ran. In the GTP-bound Ran, the intimate association of switch I with the nucleotide displaces the C-terminal extension from its position in contact with the G-domain in the GDP-bound form. The released C-terminal extension of Ran-GTP intimately wraps around RanBD in the complex with RanBD1 of RanBP2 [46], or becomes largely flexible and disordered in the complex with importin-β [47] or transportin [48]. Thus, the conformation of the C-terminal extension of Ran in the GTP-bound form is determined by its binding partners.

Although the use of slowly hydrolysable analogue of GTP is an effective strategy to stabilize Ran in the GTP-bound state, another strategy is to use mutants of Ran. There are Ran mutants that proved to be useful for crystallization of Ran-GTP-containing complexes (such as the nuclear export complex). A glutamine residue (Gln69) in the switch II region of Ran (Gln71 in Gsp1p) functions as a catalytic residue in the GTP hydrolysis reaction. Therefore, substitution of this glutamine with leucine reduces the GTPase activity, stabilizing Ran in the GTP-bound state and rendering Ran insensitive to RanGAP [49]. Another useful mutant is the C-terminal deletion mutant of Ran (RanΔC), which lacks the entire C-terminal extension (~40 amino acids) of Ran [50]. The RanΔC can bind to Kap-βs with high affinity even in its GDP-bound form [50]. Thus, RanΔC mutant is constitutively active and can adopt the GTP-bound conformation even when it is bound to GDP, probably because the deletion of the C-terminal extension abolishes the competition between the C-terminal extension and the switch I region to bind the same sites on the G-domain.

Structural studies on CAS (Cse1p): nuclear export receptor for importin-α (Kap60p)

CAS (Cse1p in yeast) is the exportin dedicated for nuclear export of the import adapter importin-α (Kap60p in yeast) [4,11,34,51-53]. Nuclear import of proteins containing the classical nuclear localization signal (NLS) [54] is mediated by importin-β (Kap95p in yeast) that binds to cargo in the cytoplasm via the importin-α adapter (Kap60p in yeast) [55-60]. Importin-α (Kap60p) has two domains, the importin-β-binding (IBB) domain [61,62] and the NLS-binding armadillo (ARM) repeat domain composed of 10 ARM repeats [63-66]. Like HEAT repeats, ARM repeat is a helical repeat motif, and tandem stacking of ARM repeats generates elongated super-helical structure [13]. The classical NLSs bind along the groove on the inner concave surface of the ARM repeat domain of importin-α (Kap60p) [11,63,65,66]. After NPC passage of the importin-α-β-cargo complex, the import complex is disassembled by Ran-GTP in the nucleus. Ran-GTP displaces the IBB domain, releasing importin-α-cargo complex from importin-β. The dissociated IBB domain has an autoinhibitory function and competes with NLSs to facilitate cargo release from importin-α [64]. The dissociation of NLSs from importin-α can also be facilitated by Nup50 (Nup2p in yeast), a multi-domain Nup localized predominantly on the nucleoplasmic face of NPCs [67-70]. After cargo release in the nucleus, the cargo-free importin-α is recycled to cytoplasm by its nuclear export receptor CAS complexed with Ran-GTP, whereas importin-β is separately recycled complexed with Ran-GTP.

Crystal structure of Cse1p (yeast CAS) in complex with its cargo Kap60p (yeast importin-α) and canine Ran-GTP (the first structure of the nuclear export complex) was determined in 2004 (Fig. 2a) [71]. This structure revealed extensive interaction interface between the three proteins. Cse1p is constructed from a right-handed superhelix of 20 HEAT repeats and wraps around both Kap60p and Ran-GTP to form an intimate contact over most of its inner surface, with direct contacts

also being made between Ran and the last ARM repeat of Kap60p. Both switch regions I and II of Ran are involved in Cse1p binding, and this is possible only when Ran adopts the GTP-bound conformation. The interface between Cse1p and Kap60p includes not only the ARM repeat domain but also the IBB domain of Kap60p. Importantly, Cse1p clamps the IBB domain close to the NLS-binding sites and thereby stabilizes the binding of the pseudo-NLS sequences of the IBB domain to the major and minor NLS binding sites on the ARM repeat domain. Thus, the assembly of the Cse1p-Kap60p-Ran-GTP complex reinforces the autoinhibitory activity of the IBB domain and ensures only cargo-free Kap60p is exported to the cytoplasm.

The intimate interaction interface between Cse1p and Ran is remarkably extensive when Cse1p binds both Kap60p and Ran-GTP. This extensiveness of this interface is similar to that of the interface between importin and Ran observed in the structures of importin-β-Ran-GTP complex or transportin-Ran-GTP complex [47,48]. However, in contrast to importin-β or transportin that binds Ran-GTP with high affinity, Cse1p does not bind Ran-GTP tightly in the absence of Kap60p. Therefore, the structure of the Cse1p-Kap60p-Ran-GTP complex immediately suggested that, in the absence of Kap60p and Ran, Cse1p probably adopts a conformation that is different from that observed in the export complex, and that the free energy of Kap60p binding is required to distort Cse1p to a higher energy, strained conformation that is able to bind Ran-GTP (the "spring-loading" hypothesis) [71], although it was impossible to predict the precise structure of free Cse1p from the structure of Cse1p in the export complex. Strong support for this hypothesis was soon obtained from the crystal structure of free Cse1p published in the next year [72] (Fig. 2b) that showed that, free Cse1p adopts a closed conformation [72], in contrast to a more extended and open conformation observed in the Cse1p-Kap60p-Ran-GTP complex [71], and thus is incompatible with Ran-GTP binding in the absence of Kap60p. Molecular dynamics (MD) simulations

also supported the idea that free Cse1p represents a low-energy state and that Cse1p in the export complex represents a high-energy state [73].

The structure of the Cse1p-Kap60p-Ran-GTP complex [71] also accounted for the ability of RanBP1 (Yrb1p) to disassemble the export complex in the cytoplasm [27]. Although the binding site for the RanBD of RanBP1 (Yrb1p) on the G-domain of Ran is fully exposed in the Cse1p-Kap60p-Ran-GTP complex, the association of RanBD to Ran-GTP recruits the C-terminal extension of Ran [46], which would clash with Cse1p and thereby destabilize the export complex [71].

An open question still remains as to how the Cse1p-Kap60p-Ran-GTP complex interacts with FG-repeat Nups during NPC passage. MD simulations of the binding of FG-repeat peptides diffusing through aqueous environment around the Cse1p-Kap60p-Ran-GTP complex suggested multiple binding spots of FG-repeats on the surface of the export complex (14 spots on Cse1p surface and 5 spots on Kap60p surface) [74]. Although these hypothetical binding spots for FG-repeats need to be verified experimentally, the presence of numerous binding sites for FG-repeats on the outer surface of HEAT repeats has also been suggested for importin-β (Kap95p) [75-79], and may be the general feature of Kap-βs.

Structural studies on exportin-t and exportin-5: nuclear export receptors for small structured RNAs

Exportin-t (abbreviated as Xpo-t; Los1p in yeast) and exportin-5 (abbreviated as Exp-5) are nuclear export receptors for small structured RNAs that are appropriately processed in the nucleus [80]. Xpo-t (Los1p) mediates nuclear export of tRNAs [35,81,82], whereas Exp-5 mediates nuclear export of pre-microRNAs and can also

export other small RNAs such as tRNAs, human Y1 RNA, and adenovirus VA1 RNA [36,83-87].

Analogous to the Cse1p-Kap60p-Ran-GTP complex, crystal structures of the Xpo-t-tRNA-Ran-GTP complex (Fig. 3a) [88] and the Exp-5-pre-microRNA-Ran-GTP complex (Fig. 3b) [89] showed that exportin wraps around both Ran-GTP and cargo on the inner concave surface of the HEAT repeats, with the basic surface patch of Ran making direct contact with negatively charged region of cargo. The structures advanced understanding of how these exportins recognize RNA cargoes specifically. In the Xpo-t nuclear export complex [88], Xpo-t wraps around a double-stranded RNA helix (the "acceptor stem" containing the 5' and 3' ends), which constitutes one arm of the L-shaped tRNA, and makes intimate contacts with the 3' CCA terminus on the one end of the acceptor stem, and also with the D and TΨC loops located on the other end of the acceptor stem (at the "corner" of the L) of tRNA. The 3' CCA component of the acceptor stem binds in a groove located at the N-terminal arch of Xpo-t, whereas the D and TΨC loops pack against the inner surface of the C-terminal arch of Xpo-t. This mode of specific tRNA recognition is possible only when tRNA is properly processed and folded. However, except for the contacts with the 3' CCA component and the D and TΨC loops, Xpo-t does not directly bind the base of tRNA, and the rest of the contacts are made between basic residues of Xpo-t and the negatively charged phosphate groups in the backbone of tRNA. Thus, it appears that Xpo-t specifically bind tRNA by recognizing the surface charge and correctly folded shape of tRNA, rather than by directly "reading" the sequence of tRNA. This explains why Xpo-t can bind a range of different tRNAs. In the Exp-5 nuclear export complex [89], Exp-5 adopts a U-shaped conformation and wraps around the double-stranded stem of pre-microRNA by contacting the phosphate groups of RNA backbone, and also binds the 2-nucleotide 3' overhang at one end of the stem that snugly fits into the hole located at the central

region of Exp-5. The other end of the stem protrudes out of Exp-5. The structure suggests that RNA recognition by Exp-5 does not depend on RNA sequence, but RNA cargo for Exp-5 needs to have 3' single stranded overhang and double-stranded stem (~17 bp) for efficient binding to Exp-5. Thus, like Xpo-t, Exp-5 recognizes RNA cargo through charge complementarity and shape complementarity between the exportin and RNA, although the orientation of the double-stranded RNA stem in the Exp-5 complex is opposite to that in the Xpo-t complex.

Crystal structure of free Xpo-t suggested that free Xpo-t adopts a rather open conformation that is incompatible with stable binding to tRNA and Ran-GTP [88], and it seems likely that Xpo-t undergoes an open-to-closed conformational change when Xpo-t associates with Ran-GTP and tRNA in the nucleus. Thus, Xpo-t is similar to Cse1p in that the conformational flexibility of HEAT repeats is important for cooperative assembly of the export complex. Although the direction of the unbound-to-bound conformational change is opposite (unbound Cse1p is more "closed" compared with the bound state, whereas unbound Xpo-t is more "open" compared with the bound state), this appears to be a variation of a common theme, and the key common concept is that exportin is "spring-loaded" and is in a higher energy state in the fully assembled export complex. However, one difference between Xpo-t and Cse1p is that Xpo-t forms a stable binary complex with Ran-GTP [88], whereas Cse1p does not bind tightly to Ran-GTP in the absence of cargo. Thus the assembly of Xpo-t export complex may occur in two steps. Ran-GTP would first bind Xpo-t, inducing open-to-closed conformational change of Xpo-t. The Xpo-t-Ran-GTP complex would then bind tRNA tightly. The structure of free Exp-5 is not yet available, and the mechanism of cooperative assembly of the Exp-5 export complex is not fully understood. However, it appears that Exp-5 can also form a stable complex with Ran-GTP in the absence of RNA [42], and so the assembly of the Exp-5 export complex

may also proceed through an Exp-5-Ran-GTP intermediate.

In the cytoplasm, RanBP1 and RanGAP promote release of tRNA from Xpo-t and GTP hydrolysis by Ran [81]. The structure of the Xpo-t-tRNA-Ran-GTP complex suggests that the binding of the RanBD of RanBP1 or RanBP2 would cause steric clash between Xpo-t and the C-terminal tail of Ran [42,90]. Thus, the disassembly mechanism of the Xpo-t export complex may be similar to that of the Cse1p export complex [71]. The mechanism of disassembly of the Exp-5 export complex in the cytoplasm remains poorly understood. An interesting suggestion from the structure of the Exp-5-pre-microRNA-Ran-GTP complex was that RanBD alone would not be sufficient to disassemble this export complex [42,90]. This may be important to protect pre-microRNAs from degradation until the export complex encounters the RNA processing machinery for the final maturation step of microRNAs in the cytoplasm. It may be worthwhile to try to identify cytoplasmic RNA-binding proteins that act on the Exp-5 nuclear export complex and thereby increase the off-rate of pre-microRNAs.

Structural studies on CRM1 (Xpo1p): the most versatile nuclear export receptor for many proteins and ribonucleoproteins

CRM1 (for chromosome region maintenance 1 [91]; Xpo1p in yeast) is the major exportin that mediates a broad range of proteins and ribonucleoproteins. CRM1 is outstanding among exportins in terms of the number and variety of cargo. The CRM1 cargoes include a plethora of transcription factors, signaling molecules, cell cycle regulators, ribosomal subunits, uridine-rich small nuclear RNPs (U snRNPs), a subset of mRNAs, and viral proteins and RNAs. The majority of CRM1 cargoes contain nuclear export signals (NESs) known as leucine-rich NESs [32,33,92,93]. The leucine-rich NESs are short stretches (8-15 amino acids) of leucine-rich sequences that were first

discovered in human immunodeficiency virus type 1 (HIV-1) Rev protein [94] and cAMP-dependent protein kinase inhibitor (PKI) [95]. In the case of RNA export by CRM1, NES-containing proteins function as adaptors between CRM1 and cargo RNAs. The traditional definition of the consensus sequence of the leucine-rich NESs, based on comparison of functional NESs and in vivo randomization-selection assay was Φ-x_{2-3}-Φ-x_{2-3}-Φ-x-Φ (Φ = L, I, V, F or M; x is any amino acid) [96-98], but this consensus sequence motif was recently expanded by a random peptide library screening into six patterns according to the conserved spacing between the hydrophobic residues, which may help identify functional NESs [99]. NES-containing proteins are involved in numerous cellular and disease processes, and information of experimentally validated NES-containing proteins are archived in databases such as NESdb and ValidNESs that are updated regularly [100,101].

Among CRM1 cargoes, snurportin1 (Spn1; the import adaptor for U snRNPs) has unusually high affinity to CRM1 [102], and has been useful for structural study of the mechanism of cargo recognition by CRM1 [103-105]. Crystal structures of Spn1 in binary complex with human CRM1 [103] or in ternary complex with mouse CRM1 and Ran-GTP [104] showed that Spn1 binds to CRM1 in a multipartite manner. CRM1 is a ring-shaped molecule constructed from 21 HEAT repeats, and Spn1 binds to the outer surface of the CRM1 ring. In addition to the import-cargo-binding domain of Spn1 that binds to CRM1 HEAT repeats 12-14, Spn1 has a leucine-rich NES at its N-terminus that adopts a combined α-helical-extended conformation and binds to a groove formed on the outer convex surface of CRM1 between HEAT repeats 11 and 12, with the hydrophobic side chains of the Spn1 NES fitting snugly into five hydrophobic pockets within this narrow groove. By using a chimera of Spn1 in which the N-terminal NES of Spn1 is replaced with the NES of Rev or PKI, it was shown that these NESs invariably bind to the same hydrophobic groove between HEAT repeats 11 and 12 [105]. The

binding of PKI NES to this site was recently confirmed in a high-resolution structure of Xpo1p-PKI-Gsp1p-GTP complex (Fig. 4a), in which PKI NES was not artificially tethered to the import-cargo-binding domain of Spn1 [106]. In all of the currently available structures of CRM1 (Xpo1p) in complex with NES-cargo, the conformation of the NES-binding cleft is essentially identical [103-106]. This indicates that diverse NESs adapt structurally to fit into a structurally invariant binding site. The idea that the hydrophobic groove between HEAT repeats 11 and 12 is the general binding site for the leucine-rich NESs is also supported from the observation that the binding site for Leptomycin B (LMB), a potent inhibitor of CRM1-mediated nuclear export [33,107], is located in this groove [108]. The location of the NES-binding site on the outer surface of CRM1 confers functional advantage to CRM1 in that it enables CRM1 to carry a broad range of cargoes that vary greatly in size and shape. On the other hand, in the CRM1-cargo-Ran-GTP complex, Ran-GTP binds to the inner surface of CRM1, making intimate contacts with HEAT repeats 1-4, 17 and 19, and a long β-hairpin loop (referred to as HEAT9 loop because this loop is inserted between the two α-helices of HEAT repeat 9) [104-106]. The HEAT9 loop is also called acidic loop [43,44,104], because this loop contains acidic residues important for Ran-binding, like the internal loop in importin-β (Kap95p) [47,109] or transportin [48]. However, the HEAT9 loop also contains functionally important hydrophobic residues that are crucial for allosteric regulation of NES binding and release, as described below.

The CRM1 (Xpo1p) nuclear export complex [the CRM1 (Xpo1p)-cargo-Ran-GTP (Gsp1p-GTP) complex] is disassembled in the cytoplasm by a mechanism involving destabilization of the complex by RanBP1 (Yrb1p) or RanBP2, and hydrolysis of Ran-bound GTP promoted by RanGAP (Rna1p). Three important findings were obtained from FRET-based kinetic analysis of the disassembly reaction [90]. First, the CRM1 (Xpo1p) nuclear export complex is kinetically stable, and the rate

of spontaneous dissociation of NES is quite slow. Second, RanGAP (Rna1p) does not increase the off-rate of NES. Third, any of the RanBD of RanBP1 (Yrb1p) and RanBP2 can increase the off-rate of NES dramatically (by over two orders of magnitude). Thus, the first step of the export complex disassembly is probably rapid NES dissociation induced by RanBD. Crystal structure of Xpo1p in complex with Gsp1p-GTP and the RanBD of Yrb1p (an intermediate complex in the disassembly reaction; Fig. 4b) revealed an allosteric mechanism of NES release [90]. Although RanBD binds to a site that is located away from the NES-binding site, the NES-binding site adopts a closed conformation, which is incompatible with NES binding, in this disassembly intermediate. This suggests that RanBD uses a long-range allosteric communication mechanism to induce closure of the NES-binding groove. The HEAT9 loop plays an important role in this allosteric communication (Fig. 4c). Although the HEAT9 loop binds to the switch I region of Ran in the CRM1 (Xpo1p) nuclear export complex, the binding of RanBD and recruitment of the C-terminal extension of Ran on RanBD displaces the HEAT9 loop by steric clash. The displaced HEAT9 loop is in turn relocated to bind the inner surface of HEAT repeats 11 and 12, immediately behind the NES-binding site. This results in the closure of the NES-binding cleft between HEAT repeats 11 and 12 on the outer surface. The binding of RanBD also induces slight change in the superhelical shape of Xpo1p HEAT repeats 12-19, which might contribute to NES release. However, mutational analyses suggested that it is the movement of the HEAT9 loop that is primarily responsible for rapid displacement of NES by RanBD [90].

Although kinetic analyses suggested that the mechanism of RanBD-accelerated NES release is conserved from yeast to humans [90], there is a notable difference between yeast Yrb1p and mammalian RanBP1. Because the size of RanBP1 (Yrb1p) is small enough to diffuse through NPCs passively, eukaryotic cells

use active mechanism of CRM1 (Xpo1p)-mediated nuclear export of RanBP1 (Yrb1p) to ensure predominantly cytoplasmic localization of this small protein. In yeast, the RanBD of Yrb1p is responsible for Xpo1p-mediated nuclear export of Yrb1p [110]. By contrast, mammalian RanBP1 has a leucine-rich NES at its C-terminus [110], which is absent in Yrb1p, and it is this C-terminal functional NES, not RanBD, that is responsible for CRM1-mediated nuclear export of mammalian RanBP1 [111]. Although the reason for this difference between yeast and mammalian systems is not fully understood, this difference is presumably due to the difference in the kinetic stability of CRM1-RanBD-Ran-GTP complex between species. In the case of yeast proteins, the Xpo1p-RanBD^{Yrb1p}-Gsp1p-GTP complex is probably stable enough to maintain its integrity during NPC passage, although this complex is not as kinetically stable as the Xpo1p-PKI-Gsp1p-GTP complex [90,106]. In the case of mammalian proteins, the CRM1-RanBD^{RanBP1}-Ran-GTP complex appears to be quite unstable and is presumably much less stable than the CRM1-NES^{RanBP1}-Ran-GTP complex. When CRM1 and Ran-GTP bind RanBP1 via its C-terminal NES, RanBD of this RanBP1 may be unable to access Ran in this complex and so is unable to accelerate disassembly of this complex, because the length of the linker (approximately 20 amino acids) connecting RanBD and the C-terminal NES in mammalian RanBP1 may not be long enough to allow simultaneous binding of NES and RanBD to CRM1 and Ran, respectively, in the same CRM1-RanBP1-Ran-GTP complex (provided that the NES of RanBP1 binds to CRM1 in the same way as observed for PKI NES). It is therefore conceivable that mammalian CRM1-RanBP1-Ran-GTP complex (in which the C-terminal NES of RanBP1 binds to the NES-binding site of CRM1) can maintain its integrity during NPC passage, even though the isolated RanBD of mammalian RanBP1 is able to accelerate NES release dramatically [90].

The structure of Xpo1p-Yrb1p-Gsp1p-GTP complex not only revealed the

mechanism of RanBD-accelerated NES release, but also suggested a hypothesis as to why Xpo1p cannot bind NES in the absence of Gsp1p-GTP (the autoinhibition hypothesis) [90]. Because the conformation of the closed NES-binding groove in the Xpo1p-Yrb1p-Gsp1p-GTP complex was stabilized by intramolecular interactions within Xpo1p, it seemed likely that the same interactions are responsible for stabilization of the same conformation in free Xpo1p, in the absence of cargo and Gsp1p-GTP [90]. This hypothesis was supported by mutational analyses [90], and the precise mechanism of autoinhibition was elucidated by crystal structure of free Xpo1p (Fig. 5a) [112]. As predicted, the HEAT9 loop in free Xpo1p binds to the backside of the NES-binding groove, stabilizing this groove in the closed conformation in exactly the same way as observed in the Xpo1p-Yrb1p-Gsp1p-GTP complex. Furthermore, the structure of free Xpo1p revealed that the C-terminal α-helix (referred to as the C-helix) adopts dramatically different conformation compared to the Xpo1p-cargo-Gsp1p complex, and lies across the central cavity of the Xpo1p ring with its C-terminal tail interacting with the HEAT9 loop and also with the inner surface behind the NES-binding site. The C-terminal tail in free Xpo1p interacts with the HEAT9 loop in such a way that stabilizes the autoinhibitory conformation of the HEAT9 loop and thereby reinforces autoinhibiton [112]. The role of the C-terminal tail of CRM1 (Xpo1p) in autoinhibition has been supported by biochemical assays [112,113]. The structure of *Chaetomium thermophilum* CRM1 suggested that the mechanism of autoinhibition in free CRM1 (Xpo1p) is evolutionarily conserved [114]. MD simulations also supported the crucial mechanistic role of the HEAT9 loop in the conformational regulation of CRM1 (Xpo1p) [115].

The overall architecture of CRM1 (Xpo1p) in the crystal structures of free *S. cerevisiae* Xpo1p and free *C. thermophilum* CRM1 is somewhat "open" and is less compact than the cargo- and Ran (Gsp1p)-bound conformation of the CRM1 (Xpo1p)

[112,114], indicating that the binding of Ran-GTP (Gsp1p-GTP) to the interior of CRM1 (Xpo1p) induces substantial conformational changes in CRM1 (Xpo1p). One possibility is that Ran-GTP (Gsp1p-GTP) binds to CRM1 (Xpo1p) in an induced fit manner [112]: Ran-GTP (Gsp1p-GTP) would displace the C-helix from the interior of the CRM1 (Xpo1p) ring to the outside and also induce movement of the HEAT9 loop, resulting in closure of the ring and opening of the NES-binding groove, allowing for stable binding of both Ran-GTP (Gsp1p-GTP) and NES-cargoes. However, combinations of single particle electron microscopy (EM), MD simulations and small-angle X-ray scattering experiments suggested that free *C. thermophilum* CRM1 and mammalian CRM1 exist in equilibrium between two conformations: one is an extended conformation similar to the crystal structures of free CRM1, and the other is a closed-ring conformation similar to the structure of CRM1 in the crystal structures of CRM1-cargo-Ran-GTP complex [114,115]. If subpopulation of free CRM1 (Xpo1p) adopts the closed ring architecture suitable for high-affinity Ran (Gsp1p)-binding, the association kinetics of Ran (Gsp1p) to this population of free CRM1 (Xpo1p) could be fast and the binary CRM1-Ran-GTP complex (Xpo1p-Gsp1p-GTP complex) could be stable. Available biochemical data indicate that there may be differences between species in this regard [106]. The rate of association of *S. cerevisiae* Gsp1p-GTP to *S. cerevisiae* Xpo1p is extremely slow [106], and the binding of Gsp1p-GTP alone to Xpo1p is hardly detectable in a pull-down assay [106, 112]. However, the rate of association of mammalian Ran-GTP to mammalian CRM1 is biphasic and both fast and slow phases were observed, and the binding of Ran-GTP to CRM1 is readily detectable in a pull-down assay [106]. These biochemical observations could be explained if free *S. cerevisiae* Xpo1p exists only in the open state as observed in its crystal structure but free mammalian CRM1 exists in equilibrium between the open state and the closed state. In other words, the energetic penalty associated with the open-to-closed transition (i.e., the compaction of the ring) of free CRM1 (Xpo1p) may be higher for *S. cerevisiae*

Xpo1p than for mammalian CRM1.

In the yeast system, although the rate of spontaneous assembly of Xpo1p-NES-Gsp1p-GTP complex is very slow, a predominantly nuclear Ran-binding protein Yrb2p can dramatically increase the rate of association of Gsp1p-GTP to Xpo1p and also the rate of association of NES to Xpo1p in the presence of Gsp1p-GTP [106]. Although *YRB2* is not essential for yeast viability, efficient export of NES-cargoes in yeast cells requires Yrb2p [116,117]. RanBP3, the human homologue of Yrb2p, is also known as a cofactor that promotes CRM1-mediated nuclear export [118,119]. Yrb2p is a multi-domain protein containing N-terminal NLS, a central domain containing FG-repeats that bind Xpo1p specifically, and a C-terminal RanBD that is homologous to Yrb1p. However, Yrb2p binds to Gsp1p-GTP much more weakly than Yrb1p [120]. X-ray crystallography of Xpo1p-Yrb2p-Gsp1p-GTP complex (Fig. 5b) coupled with functional analyses suggested an assisted-assembly model of the Xpo1p nuclear export complex (Fig. 5c) [106]. Yrb2p functions as a scaffold to recruit Gsp1p-GTP to Xpo1p rapidly (the first step in Fig. 5c). Although the NES-binding cleft in the crystal structure of Xpo1-Yrb2p-Gsp1p-GTP complex is closed as observed in the structure of Xpo1-Yrb1p-Gsp1p-GTP complex, the association of the RanBD of Yrb2p to Gsp1p-GTP is so weak that NES can displace the RanBD rapidly. In other words, the reverse reaction of Yrb1p-accelerated cargo release can occur easily for Yrb2p. Therefore, the Xpo1-Yrb2p-Gsp1p-GTP complex can bind to NES rapidly (the second step in Fig. 5c).

The crystal structure of the Xpo1p-Yrb2p-Gsp1p-GTP complex was also noteworthy in that it revealed how FG-repeats of Yrb2p bind to Xpo1p [106]. In this structure, the phenylalanine side chains of the FG-repeats are buried in hydrophobic depressions between adjacent HEAT repeats [106]. This is analogous to the way

FG-Nups bind to importin-β [75,76,78] and implicates that importins and exportins use similar mechanism for NPC passage. Biochemical assays indicated that the FG-repeat binding sites observed in the crystal structure of the Xpo1p-Yrb2p-Gsp1p-GTP complex also serve as the general binding sites for FG-Nups [106]. However, these crystallographically observed binding sites probably represent the strong binding sites for FG-repeats, and it is quite likely that there are residual, weak binding sites for FG-repeats on the surface of Xpo1p [106]. Therefore the dissociation of FG-repeats of Yrb2p from Xpo1p may not be required for the Xpo1p nuclear export complex to dock at and move through NPCs [106]. It is conceivable that Yrb2p could speed up the NPC passage of the nuclear export complex by occluding the strong binding sites and exposing only the weak binding sites for FG-repeats, and thereby preventing the export complex from being trapped in the transport channel of NPCs. In this way, Yrb2p might be able to escort the Xpo1p nuclear export complex through NPCs rapidly, although this hypothesis remains to be tested. It is also conceivable that, if Yrb2p escorts the Xpo1p nuclear export complex to the cytoplasm, Xpo1p may come back to the nucleus as a binary complex with Yrb2p after releasing cargo and Gsp1p in the cytoplasm. In this case, the N-terminal NLS of Yrb2p would facilitate nuclear import of the Xpo1p-Yrb2p complex. Thus, it is conceivable that Yrb2p could promote the Xpo1p nuclear export cycle in many ways.

Finally, targeting nuclear transport pathways by small-molecule inhibitors to develop effective therapeutics has attracted increasing interest over the past decade, and the development of CRM1-specific inhibitors is probably the most advanced in this regard. CRM1 can mediate nuclear export of tumor suppressors such as p53, pRb, FOXO, and IkB, which inhibit cellular growth when localized to the nucleus. In several tumor cell types, CRM1 is overexpressed and can cause aberrant cytoplasmic localization and degradation of the tumor suppressors. Inhibitors of CRM1 therefore

could potentially be useful to treat cancer. Unfortunately, LMB (the naturally occurring CRM1 inhibitor that binds CRM1 irreversibly) is too cytotoxic to be used clinically [122]. This inspired development of next-generation, small-molecule inhibitors that are less cytotoxic. The KPT-SINE compounds, the reversible CRM1 inhibitors that were successfully designed to bind reversibly to the NES-binding cleft [123], have minimal cytotoxicity and is showing anti-cancer activity with good tolerability in clinical trials [39,121,124,125]. CRM1-specific inhibitors could also be potent antivirals, because many viruses hijack CRM1-mediated nuclear export pathway for their replication in the host. Indeed, recently it was shown that KPT-335 (Verdinexor), a representative KPT-SINE compound, can effectively inhibit replication of various influenza virus strains, probably by blocking CRM1-mediated nuclear export of viral genomic RNAs replicated in the nucleus [126]. Although the clinical development of the KPT-SINE compounds appears to be a successful model for translational research, development of even better inhibitors with minimal cytotoxicity should be continued. In particular, the cytotoxicity of the drugs is a serious issue in the case of human diseases that require prolonged treatment such as acquired immune deficiency syndrome (AIDS). In order to minimize cytotoxicity of the inhibitors, development of inhibitors that bind to sites distinct from the NES-binding sites may be an interesting avenue to pursue. In this context, a recent study on the assembly of the HIV RNA nuclear export complex is inspiring [127,128]. HIV uses the Rev protein to bind to and origomerize on the Rev response element (RRE) RNA to direct CRM1-mediated nuclear export of viral RNAs. Single particle EM analysis of a nuclear export complex containing Rev, RRE, and human CRM1 suggested that human CRM1 forms an ordered dimer to promote nuclear export of viral RNAs, and that the dimerization interface of CRM1 is probably the same as one of the crystal packing interfaces observed in the crystal structure of the human CRM1-Spn1 complex [103]. In this case, the structural information on the crystal contacts could be useful for rational design of novel antiretroviral drugs targeted to

disrupt higher order assembly of the nuclear export complex.

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Figure legends

Fig. 1. The conformational switches of Ran: switch I, switch II, and the C-terminal extension. (a) GDP-bound conformation of Ran (PDB code, 1BYU) [129]. (b) GTP-bound conformation of Ran in transportin-Ran-GppNHp complex (PDB code, 1QBK) [48]. The bound nucleotides are shown as spacefilling spheres throughout this review. Ran is colored cyan, with its switch I, switch II, and the C-terminal extension highlighted in pink, gray, and blue, respectively. This coloring of Ran is used throughout this review. The structural figures in this review were produced using Molscript [130] and Raster3D [131].

Fig. 2. The conformation of the exportin Cse1p changes substantially upon cooperative binding of cargo (Kap60p) and Ran-GTP. (a) Structure of Cse1p (yellow) in complex with Kap60p (green, except for the IBB domain that is colored magenta) and Ran-GTP (PDB code, 1WA5) [71]. (b) Structure of free Cse1p (PDB code, 1Z3H) [72], which is shown in the same view as in (a), after superposition of the N-terminal arch of Cse1p (HEAT repeats 1-7).

Fig. 3. The nuclear export complexes for small RNAs. (a) Structure of Xpo-t (yellow) in complex with tRNA and Ran-GTP (PDB code, 3ICQ) [88]. (b) Structure of Exp-5 (yellow) in complex with pre-microRNA and Ran-GTP (PDB code, 3A6P) [89]. The RNAs are shown in ball-and-stick representation with magenta carbons.

Fig. 4. Structures of the Xpo1p (yeast CRM1) nuclear export complex and its disassembly intermediate. (a) Structure of Xpo1p (yellow) in complex with PKI (purple) and Gsp1p-GTP (PDB code, 3WYG) [106]. The HEAT9 loop is highlighted in magenta. (b) Structure of Xpo1p (yellow) in complex with Yrb1p (green) and

Gsp1p-GTP (PDB code, 3M1I) [90], which is shown in the same view as in (a), after superposition of the G-domain of Gsp1p. (c) Schematic illustration the allosteric mechanism whereby RanBD of RanBP1 (Yrb1p) or RanBP2 accelerates dissociation of NES-cargo in the cytoplasm [90]. See text for details.

Fig. 5. Assembly of the Xpo1p nuclear export complex assisted by Yrb2p in the nucleus. (a) Structure of free Xpo1p (PDB code, 3VYC) [112]. The HEAT9 loop and the C-helix are highlighted in magenta and green, respectively. (b) Structure of Xpo1p (yellow) in complex with Yrb2p (red) and Gsp1p-GTP (PDB code, 3WYF), an assembly intermediate that can bind NES-cargo rapidly in the nucleus [106]. The phenylalanine side chains of the two FG-repeats (FG1 and FG2) of Yrb2p that bind to the outer surface of Xpo1p are shown in ball-and-stick representation with red carbons. (c) Schematic illustration of how Yrb2p accelerates assembly of the Xpo1p nuclear export complex in the nucleus [106]. See text for details.

Figure 1

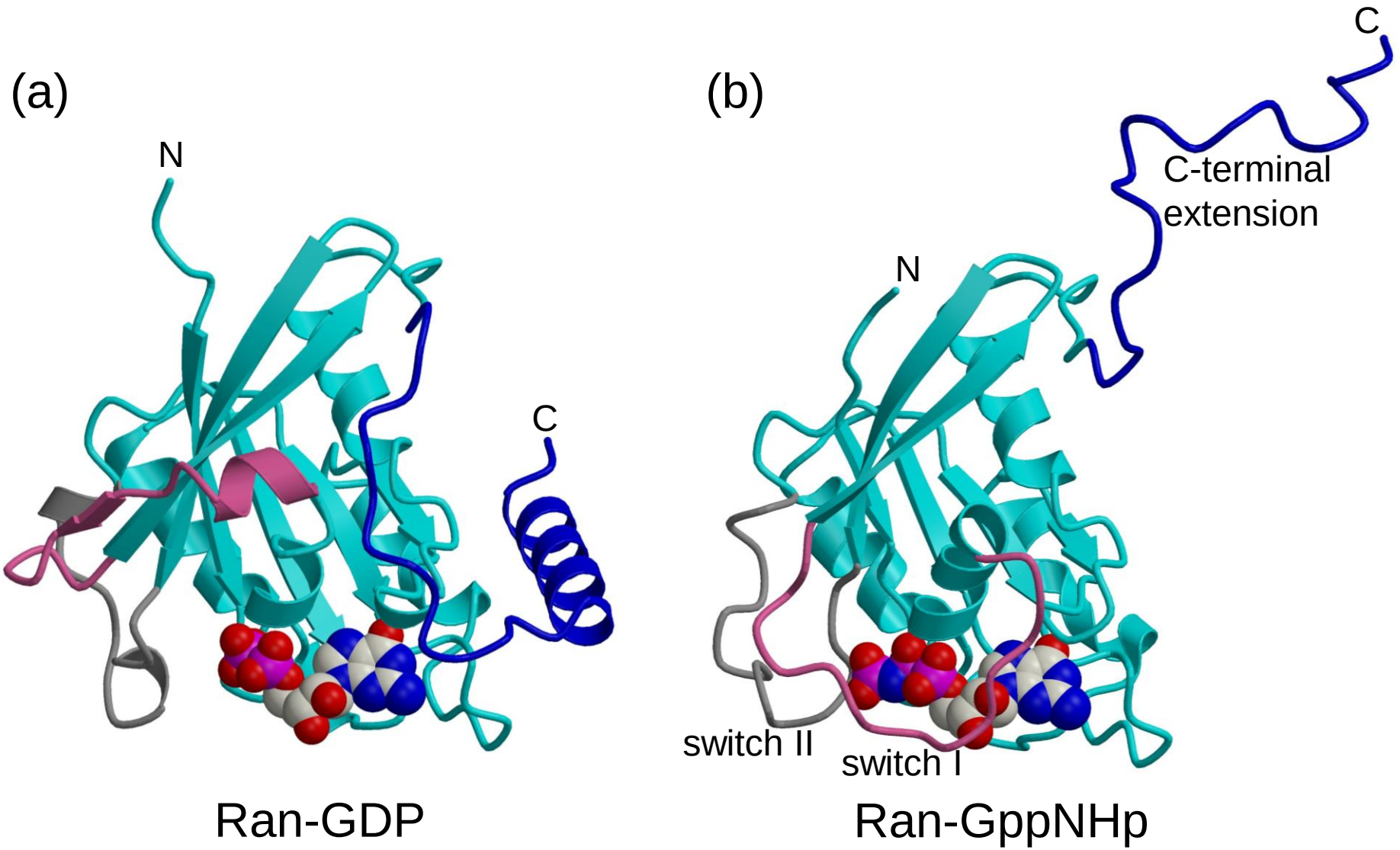

(a)
N
C
Ran-GDP
(b)
C
C-terminal
extension
N
switch II
switch I
Ran-GppNHp

Figure 2

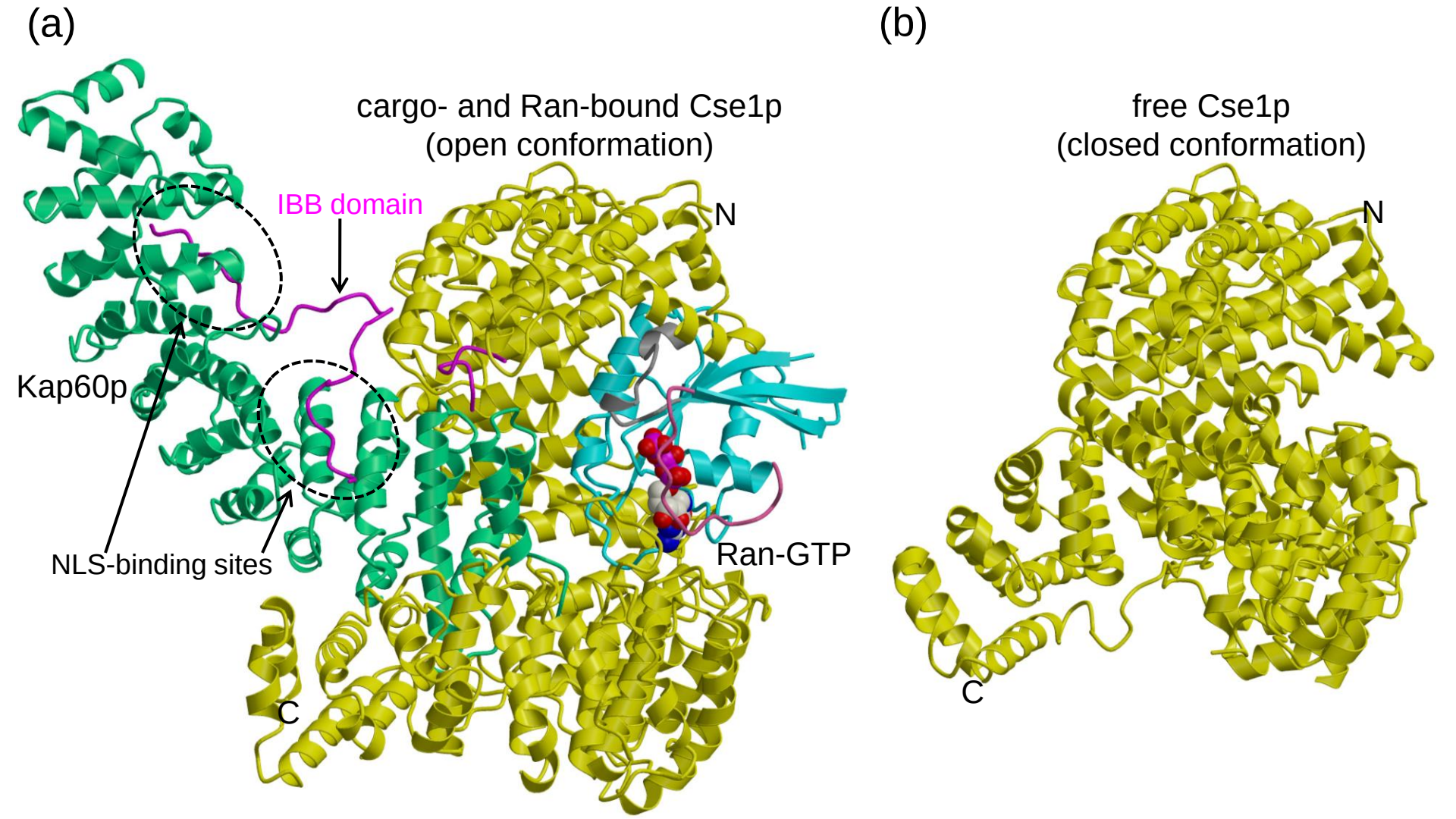

(a)
cargo- and Ran-bound Cse1p
(open conformation)
IBB domain
N
Kap60p
NLS-binding sites
Ran-GTP
C
(b)
free Cse1p
(closed conformation)
N
C

Figure 3

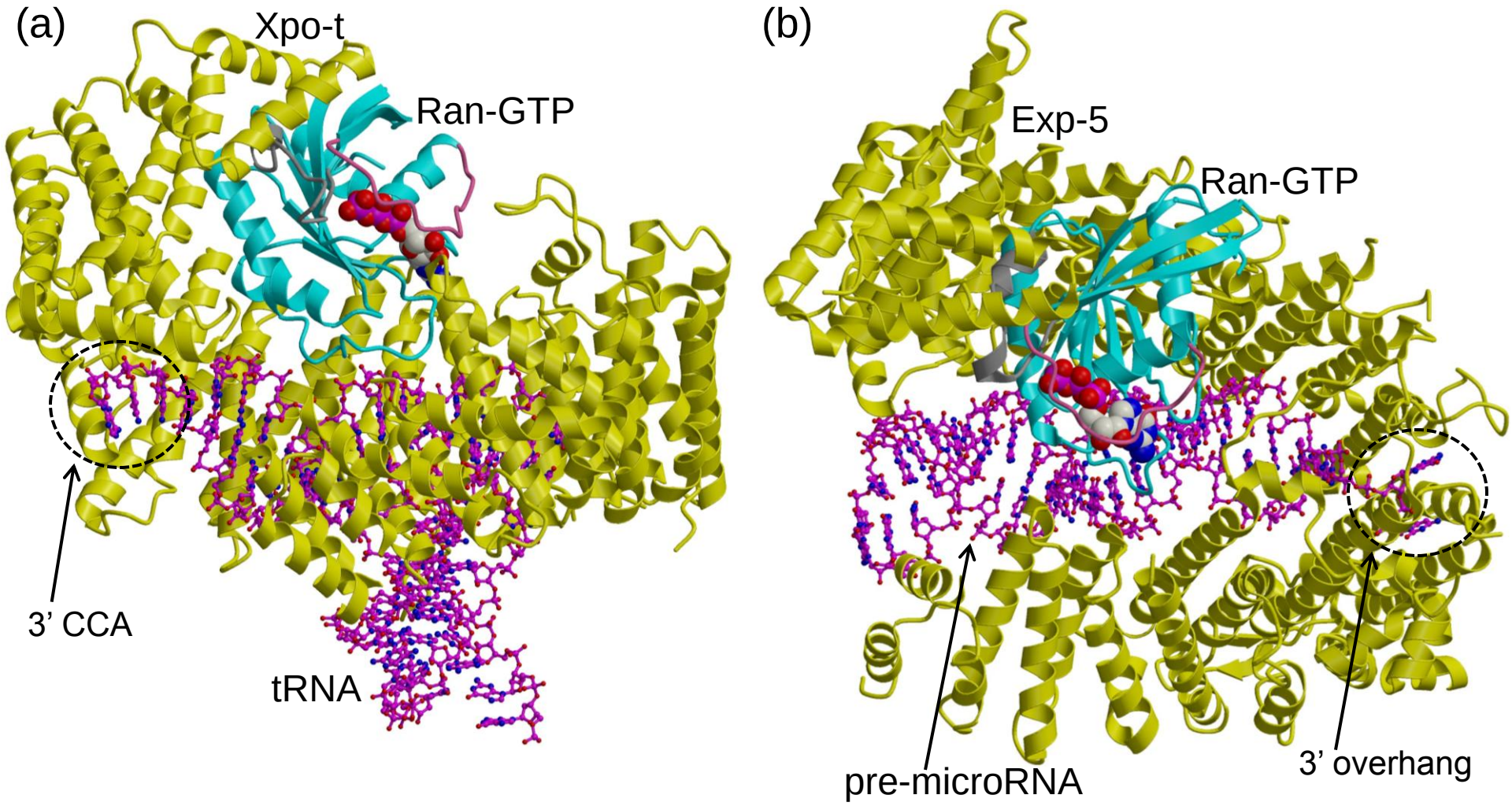

(a)
Xpo-t
Ran-GTP
3’ CCA
tRNA
(b)
Exp-5
Ran-GTP
pre-microRNA
3’ overhang

Figure 4

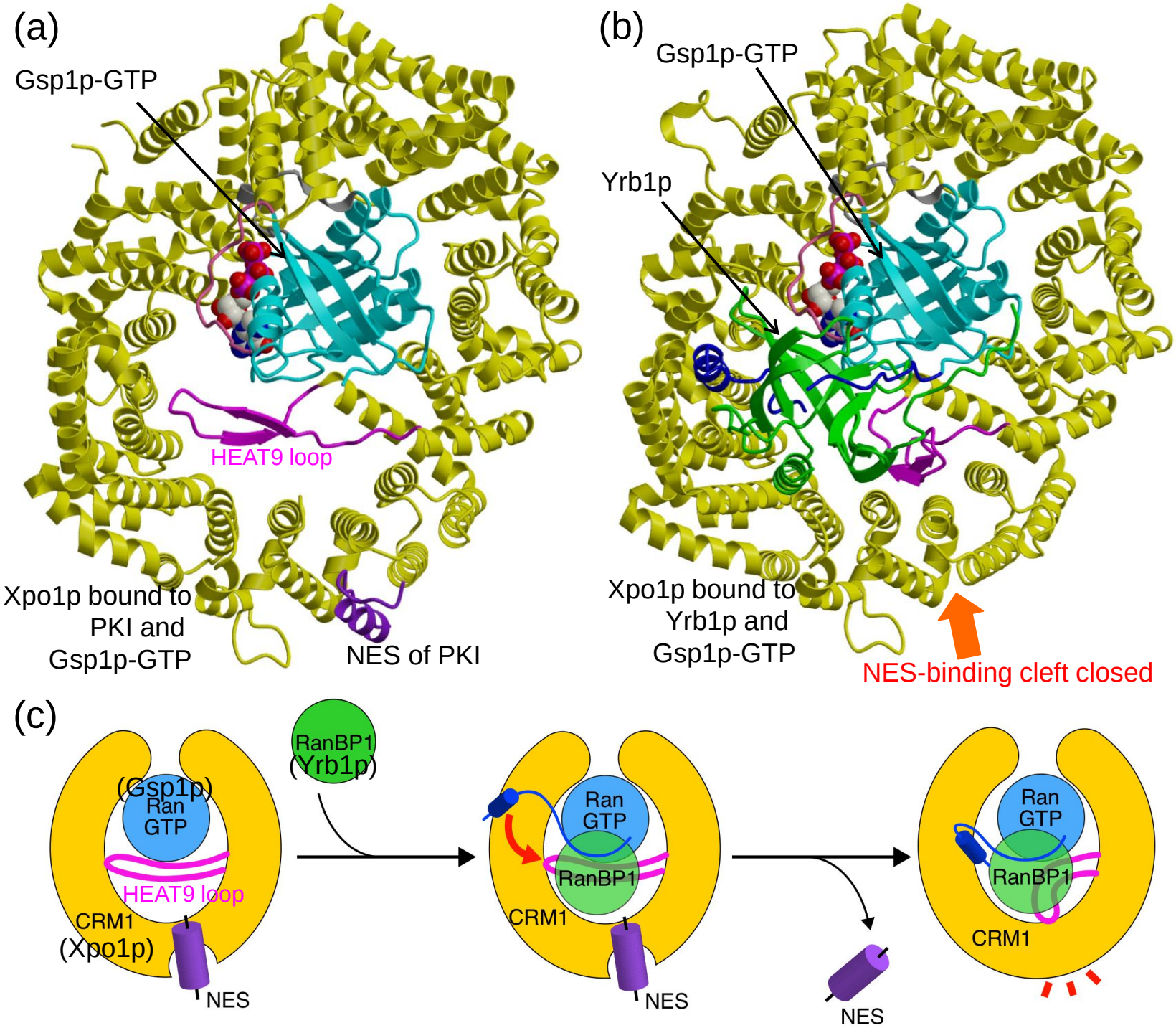
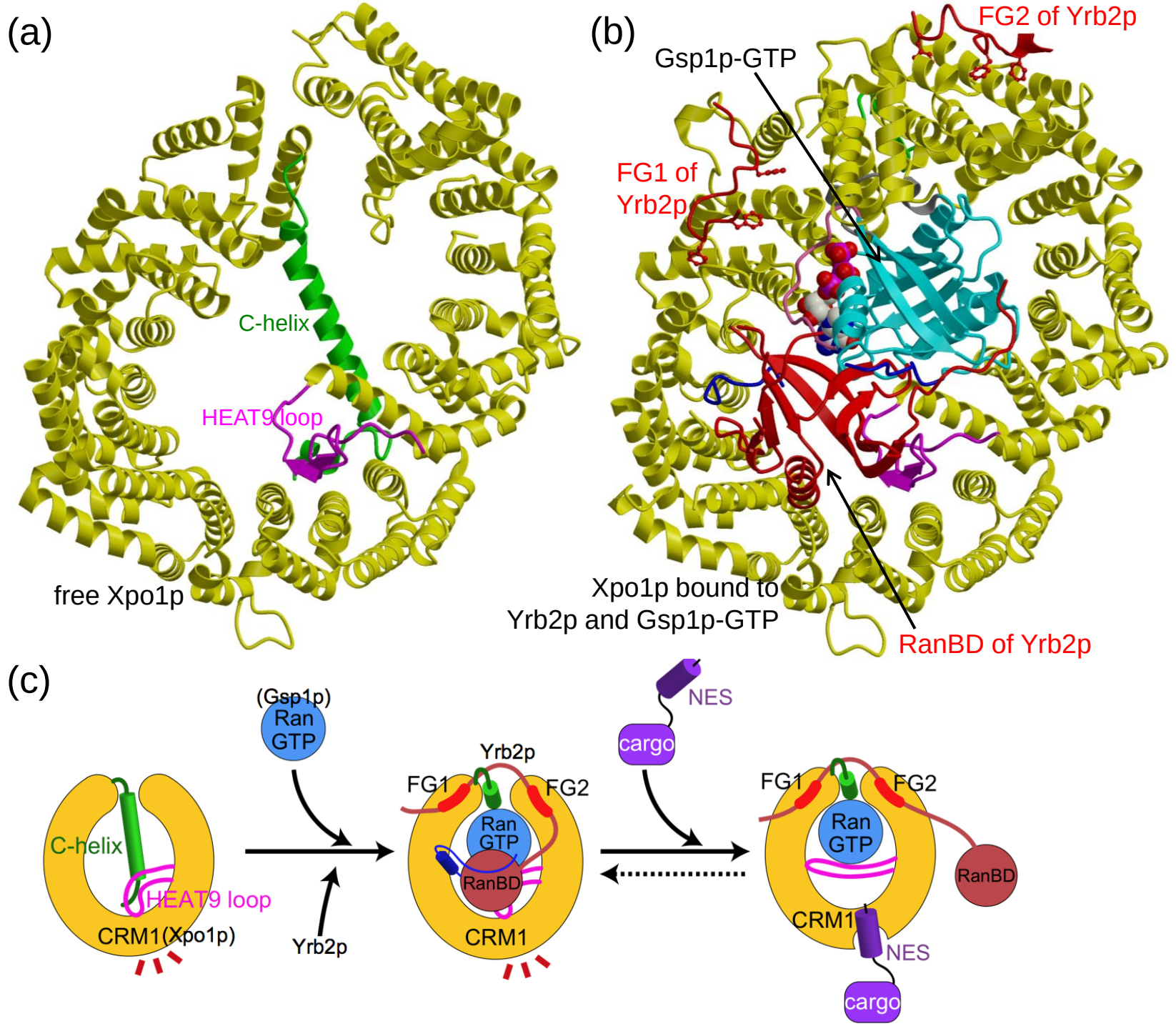

(a)
Gsp1p-GTP
HEAT9 loop
Xpo1p bound to
PKI and
Gsp1p-GTP
NES of PKI
(b)
Gsp1p-GTP
Yrb1p
Xpo1p bound to
Yrb1p and
Gsp1p-GTP
NES-binding cleft closed
(c)
(Gsp1p)
Ran
GTP
HEAT9 loop
CRM1
(Xpo1p)
NES
RanBP1
(Yrb1p)
Ran
GTP
RanBP1
CRM1
NES
NES
Ran
GTP
RanBP1
CRM1

Figure 5


(a)
C-helix
HEAT9 loop
free Xpo1p
(b)
FG2 of Yrb2p
Gsp1p-GTP
FG1 of Yrb2p
Xpo1p bound to Yrb2p and Gsp1p-GTP
RanBD of Yrb2p
(c)
(Gsp1p)
Ran GTP
C-helix
HEAT9 loop
CRM1(Xpo1p)
Yrb2p
Yrb2p
FG1
FG2
Ran GTP
RanBD
CRM1
NES
cargo
FG1
FG2
Ran GTP
RanBD
CRM1
NES
cargo