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Allosteric control of the exportin CRM1 unraveled by crystal structure analysis

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Nucleocytoplasmic trafficking in eukaryotic cells is a highly regulated and coordinated process which involves an increasing variety of soluble nuclear transport receptors. Generally, transport receptors specifically bind their cargo and facilitate its transition through nuclear pore complexes, aqueous channels connecting the two compartments. Directionality of such transport events by receptors of the importin β superfamily requires the interaction with the small GTPase Ras-related nuclear antigen (Ran). While importins need RanGTP to release their cargo in the nucleus and thus to terminate import, exportins recruit cargo in the RanGTP-bound state. The exportin chromosome region maintenance 1 (CRM1) is a highly versatile transport receptor that exports a plethora of different protein and RNP cargoes. Moreover, binding of RanGTP and of cargo to CRM1 are highly cooperative events despite the fact that cargo and RanGTP do not interact directly in crystal structures of assembled export complexes. Integrative approaches have recently unraveled the individual steps of the CRM1 transport cycle at a structural level and explained how the HEAT-repeat architecture of CRM1 provides a framework for the key elements to mediate allosteric interactions with RanGTP, Ran binding proteins and cargo. Moreover, during the last decade, CRM1 has become a more and more appreciated target for anti-cancer drugs. Hence, detailed understanding of the flexibility, the regulatory features and the positive binding cooperativity between CRM1, Ran and cargo is a prerequisite for the development of highly effective drugs. Here we review recent structural advances in the characterization of CRM1 and CRM1-containing complexes with a special emphasis on X-ray crystallographic studies.

Introduction

Membrane enclosed cellular compartments of eukaryotes like the nucleus, mitochondria or plastids allow a strict spatiotemporal separation of cellular processes. Beyond the pure functional division, compartmentalization allows concentration gradients of ions or molecules to be generated and separate reaction spaces to

Abbreviations
CBD, cap binding domain; CRIME, CRM1-importin β etc.; CRM1, chromosome region maintenance 1; EM, electron microscopy; HEAT, huntingtin, elongation factor 3 (EF3), the regulatory A subunit of protein phosphatase 2A (PP2A) and the P3 kinase TOR1; LMB, leptomycin B; MD, molecular dynamics; NES, nuclear export signal; NPC, nuclear pore complex; Nup, nucleoporin; PHAX, phosphorylated adapter for RNA export; PKI, protein kinase inhibitor; RanBP, Ran binding protein; RanGAP1, Ran GTPase activating protein; Ran, Ras-related nuclear antigen; Rev, regulator of expression of virion proteins; SINE, selective inhibitor of nuclear export; snRNA, small nuclear RNA; SPN1, snurportin1; UsnRNP, uridine-rich small nuclear ribonucleoprotein.

be established. Hence, compartmentalization poses regulatory advantages; however, it also represents a formidable challenge, namely to ensure the efficient exchange of macromolecules between the individual compartments, e.g. the nucleus and the cytoplasm.

Besides export of most mRNAs and import of RanGDP, active transport of macromolecules across the double-layered nuclear envelope is mediated by soluble nuclear transport receptors of the importin β superfamily termed importins or exportins [1,2]. An intrinsic property of importin β type nuclear transport receptors is their ability to bind both cargo [3,4] and/or the small GTPase Ras-related nuclear antigen (Ran) in its GTP-bound form [5,6]. Importins recruit their cargo in the cytoplasm and, as binary complexes, pass the nuclear pore into the nucleus, where RanGTP binding promotes cargo release and import termination (Fig. 1) [5,6]. Conversely, exportins bind cargo and RanGTP cooperatively in the nucleus to form a functional ternary export complex, which translocates to the cytoplasm [3,4]. Here, additional factors like Ran binding proteins (e.g. RanBP1 and RanBP2), the Ran GTPase activating protein (RanGAP1) or nucleoporins (Nups) like Nup214 support cargo release and facilitate GTP hydrolysis by Ran (Fig. 1). The RanGTP gradient across the nuclear envelope with a 200–1000-fold higher RanGTP concentration in the nucleus [7–9] enables directionality of transport and allows the transport receptors to act as highly efficient unidirectional cargo pumps [6,10]. The RanGTP gradient itself is maintained by the asymmetric distribution of Ran regulatory factors, namely RanGAP1 in the cytoplasm [11–13] and the Ran guanine nucleotide exchange factor in the nucleus (Fig. 1) [14,15].

The exportin chromosome region maintenance 1 (CRM1) mediates export of a plethora of functionally and structurally unrelated protein and RNP cargoes [16,17] and was found to be essential in all organisms tested so far. For example, CRM1 depletes several proteins from cell nuclei including the import adapter snurportin1 (SPN1) [18] as well as a set of eukaryotic translation initiation and termination factors [19]. Apart from this function, CRM1 is strictly required for several viral lifecycles. For example, export of the unspliced HIV-1 mRNA depends on CRM1 [3,20–22]. Moreover, export of several cellular mRNAs, like COX-2, c-Fos, cyclin D1 [23–26], and of diverse RNPs including the small and large ribosomal subunit and the signal recognition particle is mediated by CRM1 [27–29].

In recent years, great progress has been made by several groups towards a complete understanding of the structure–function relation of CRM1 at an atomic level. In this review we focus on the recent advances in the structural characterization of CRM1 and CRM1-containing complexes with a special emphasis on

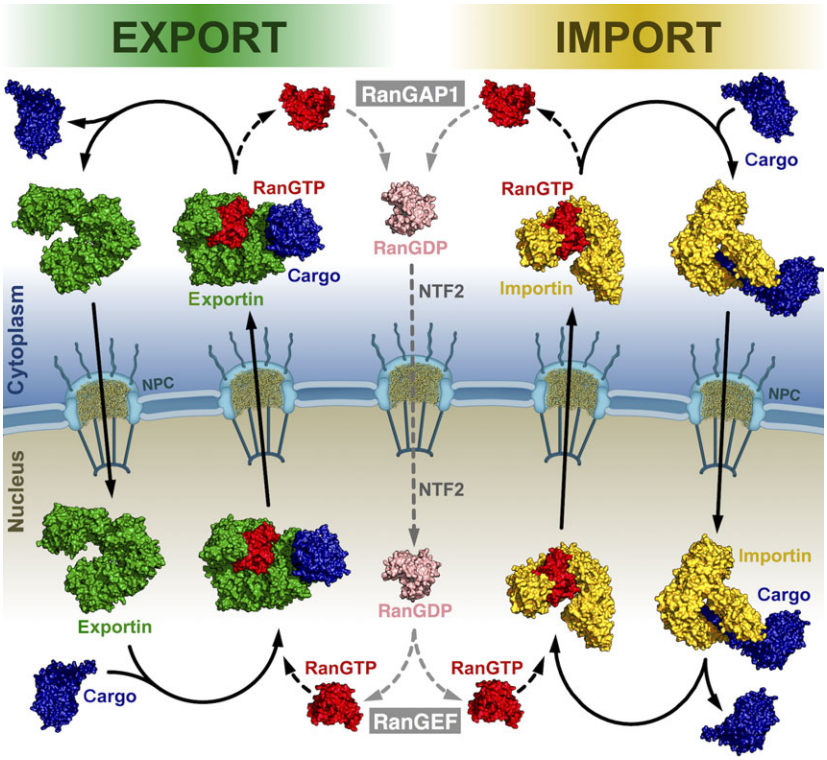


Fig. 1. Schematic overview of nuclear export (left) and nuclear import (right) by importin β type transport receptors through nuclear pore complexes (NPCs). Exportins (green) need RanGTP (red) to recruit cargo (blue) in the nucleus while importin-cargo complexes are dissociated by RanGTP binding (import termination). Note the opposite function of RanGTP in nuclear import and export. The cytoplasmic Ran GTPase activating protein (RanGAP1) constantly hydrolyzes RanGTP to RanGDP, which is actively transported back to the nucleus by the nuclear transport factor 2 (NTF2). The Ran guanine nucleotide exchange factor (RanGEF) ensures a constant high RanGTP concentration in the nucleus.

X-ray crystallography and provide an outlook to the future challenges.

Cellular functions of CRM1

CRM1 was first identified as a gene mutated in cold-sensitive strains of the budding yeast *Schizosaccharomyces pombe*, which exhibits deformed chromosomes at restrictive temperatures [30]. Subsequent investigations demonstrated that mutations in the *crm1* gene trigger the deregulation of a transcription factor, namely pap1 (homologue of human transcription factor AP1) [31], and may lead to multiple drug resistances [32,33]. Later CRM1 was found to interact with a protein of the nuclear pore complex (NPC), Can/Nup214 [34,35], suggestive of a role as a nuclear transport receptor. CRM1 function is highly conserved in eukaryotes, as human CRM1 has the ability – at least in part – to complement a yeast defect [36]. *In vitro* and *in vivo* experiments clearly demonstrated its role as an export receptor [3,37–41] and identified the cargoes as proteins bearing a so-called classical or leucine-rich nuclear export signal (NES) [21,22]. For more complicated export events additional factors and adapters may be necessary. For example, export of m^7G-capped small nuclear RNAs (snRNAs) additionally requires PHAX (phosphorylated adapter of RNA export) as well as the cap binding complex composed of the cap binding proteins 20 and 80 [42,43].

In recent years, CRM1 has been identified as a binding partner of various (proto-) oncogenes like p53, Rb, BRCA1, p21/CIP, p27/KIP1 and cyclin D1 [44–48], making it an interesting target in molecular oncology and therapeutics. The identification of the natural CRM1-specific export inhibitor leptomycin B (LMB) [3,36,37,49], the recent structural understanding of cargo recognition by CRM1 within the NES cleft and the blockade of exactly that binding cleft by LMB [50–52] led to the development of novel therapeutics [53–55]. These compounds block CRM1-mediated export, and by influencing the nucleocytoplasmic distribution pattern of the oncogenes they modulate the fate of cancer cells by decreasing their survival rate [54–61]. With respect to the growing interest in medical research towards CRM1 and its role related to various cancers such as breast cancer, pancreatic cancer, leukemia and lymphoma [44,54,55,57–59,61–63] it is important to understand also the dynamics of mammalian CRM1 with a special emphasis on the NES cleft, where the therapeutic compounds bind.

Later investigations manifested additional roles of CRM1 in different cellular processes, e.g. opposing the effects of importin β in mitosis [64]. Moreover, CRM1 plays a role in mitotic progression as it localizes to kinetochores and binds RanGAP1 and RanBP2 in a RanGTP-dependent manner. In addition, CRM1 affects the definition of kinetochore fibers and is involved in chromosome segregation at mitosis. In particular, CRM1 activity in the metaphase and late anaphase changes repartitioning of RanGTP and thus of effectors on kinetochores and centrosomes [64–69]. While CRM1 function in all the latter processes remains less well understood, recent progress in structural analysis has revealed a detailed picture of the structural changes in CRM1 during the individual steps of the nuclear transport cycle.

In 2004, the crystal structure of the C-terminal third of human CRM1 (amino acids 707–1027) was published, reporting on the architecture of CRM1 HEAT-repeat helices 15A–21A [70]. To date, 26 crystal structures of free CRM1 or CRM1 in complex with different interacting proteins are available in the Protein Data Bank (PDB). For example, crystal structures of CRM1 in complexes with the cargo SPN1 (CRM1-SPN1; PDB ID 3GB8) [51], RanGTP (CRM1-RanGTP; PDB ID 3NC1) [71], cargo and RanGTP (CRM1-RanGTP-SPN1; PDB ID 3GJX) [50] as well as RanGTP and the cytoplasmic release trigger factor RanBP1 (CRM1-RanGTP-RanBP1; PDB ID 3M1I) [72] are available (Table 1). In addition to the relatively rigid and similar conformation of CRM1 in the various complexes, X-ray crystallography, electron microscopy (EM) and small angle X-ray scattering of free CRM1 provided evidence of the great conformational flexibility of this transport receptor (PDB IDs 4FGV, 3VYC, 4BSN, 4BSM; EMD-2110, 2111, 2274, 5564) [73–76]. Moreover, molecular dynamics (MD) simulations provided insight into the structural or conformational selection and stabilization of the different binding sites by their respective binding partners [73,74].

CRM1 – structural requirements for Ran and cargo binding

Generally, importin β type nuclear transport receptors are composed of a varying number of HEAT repeats. The canonical HEAT repeat is a structural motif formed by two anti-parallel helices (named A- and B-helix) connected by a short linker loop and a typical total length of ~ 40–50 residues (Fig. 2A) [77,78]. They have been named after the first couple of proteins bearing this motif, namely huntingtin, elongation factor 3 (EF3), the regulatory A subunit of protein phosphatase 2A (PP2A) and the P3 kinase TOR1.

Table 1. Properties of all CRM1-containing crystal structures available in the Protein Data Bank. The structure completeness is the percentage of CRM1 atoms present in the coordinate file with respect to the expected total number of atoms based on each wild-type sequence. *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Sc*, *Saccharomyces cerevisiae*; *Ct*, *Chaetomium thermophilum*.

Structure	State	Resolution	PDB ID	Space group	Mol/au	Structure completeness	Reference
Export complexes							
Hs CRM1 *Hs* SPN1	Ran-free cargo complex	2.90 Å	3GB8	$P6_422$	1	88%	[51]
Mm CRM1 *Hs* SPN1 *Hs* RanGTP	Export complex	2.50 Å	3GJX	$P2_1$	2	98%	[50]
Mm CRM1 *Hs* SPN1-PKI *Hs* RanGTP	PKI-NES (SPN1 chimera)	3.42 Å	3NBY	$P2_1$	2	97%	[71]
Mm CRM1 *Hs* SPN1-Rev *Hs* RanGTP	Rev-NES (SPN1 chimera)	2.80 Å 2.90 Å	3NBZ 3NC0	$P2_1$ $P2_1$	2 2	97% 97%	
Mm CRM1 *Hs* RanGTP	Cargo-free Ran complex	3.35 Å	3NC1	$C222_1$	1	96%	
Disassembly complex							
Sc CRM1 *Sc* RanBP1 *Sc* RanGTP	Disassembly complex (Δ377–413)	2.00 Å 1.80 Å	3M1I 4HB2	$P4_32_12$ $P4_32_12$	1	94% 98%	[72] [52]
Inhibitors							
Sc CRM1[b] *Sc* RanBP1 *Sc* RanGTP	LMB bound (Δ377–413)	1.78 Å 1.90 Å 2.30 Å 2.05 Å 2.80 Å 1.90 Å 2.20 Å	4HAT 4HAW 4HAY 4HB4 4HB3[a] 4HAZ 4HB0	$P4_32_12$ $P4_32_12$ $P4_32_12$ $P4_32_12$ $P4_32_12$ $P4_32_12$ $P4_32_12$	1 1 1 1 1 1 1	97% 96% 97% 95% 95% 95% 95%	[52]
	Anguinomycin B bound (Δ377–413)	2.00 Å	4HAV	$P4_32_12$	1	97%	
	Ratjadone A bound (Δ377–413)	2.00 Å 2.28 Å	4HAU 4HAX	$P4_32_12$ $P4_32_12$	1 1	97% 97%	
	KPT185 bound (Δ377–413)	2.10 Å	4GMX	$P4_32_12$	1	97%	[54]
	KPT251 bound (Δ377–413)	2.22 Å	4GPT	$P4_32_12$	1	96%	[55]
Free CRM1							
Ct CRM1	Thermophilic CRM1	2.94 Å 3.10 Å	4FGV 4HZK	$P2_12_12_1$ $P3_1$	1 2	99% 96%	[74]
Hs CRM1	C-terminal deletion (Δ1033–1071)	4.10 Å 4.50 Å	4BSN 4BSM	$C222_1$ $C222_1$	1 1	63% 79%	[76]
Sc CRM1	(Δ377–413, Δ971–984)	2.10 Å	3VYC	$P4_1$	1	90%	[75]
Hs CRM1	HEAT helices 15A–21A (amino acids 707–1027)	2.30 Å	1W9C	$P2_12_12$	2	30%	[70]

[a] LMB in this crystal structure was weakly bound and thus not modeled.
[b] Crystal structures contain different point mutations in the NES cleft of Sc CRM1 as specified in the PDB file.

CRM1 is composed of an array of 21 such HEAT repeats forming a continuous hydrophobic core (Fig. 2B) [50]. A slight tilt between consecutive HEAT repeats and their tandem-stacking arrangement in nuclear transport receptors leads to an overall superhelical shape. Structural analysis of a variety of nuclear transport receptors revealed a lack of interactions of residues sequentially distant to each other, which is a quite common feature of globular proteins. Consequently, this arrangement provides the transport receptor with a remarkable high flexibility, as demonstrated by various crystal structures and MD simulations for the prototypical transport receptor importin β [79–84].

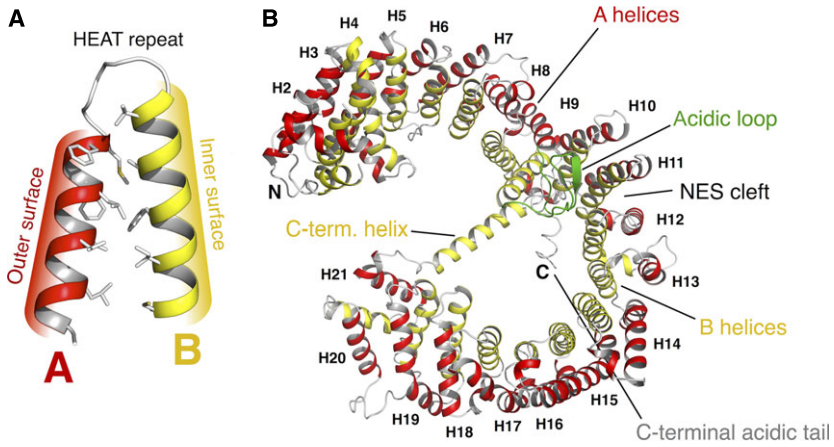
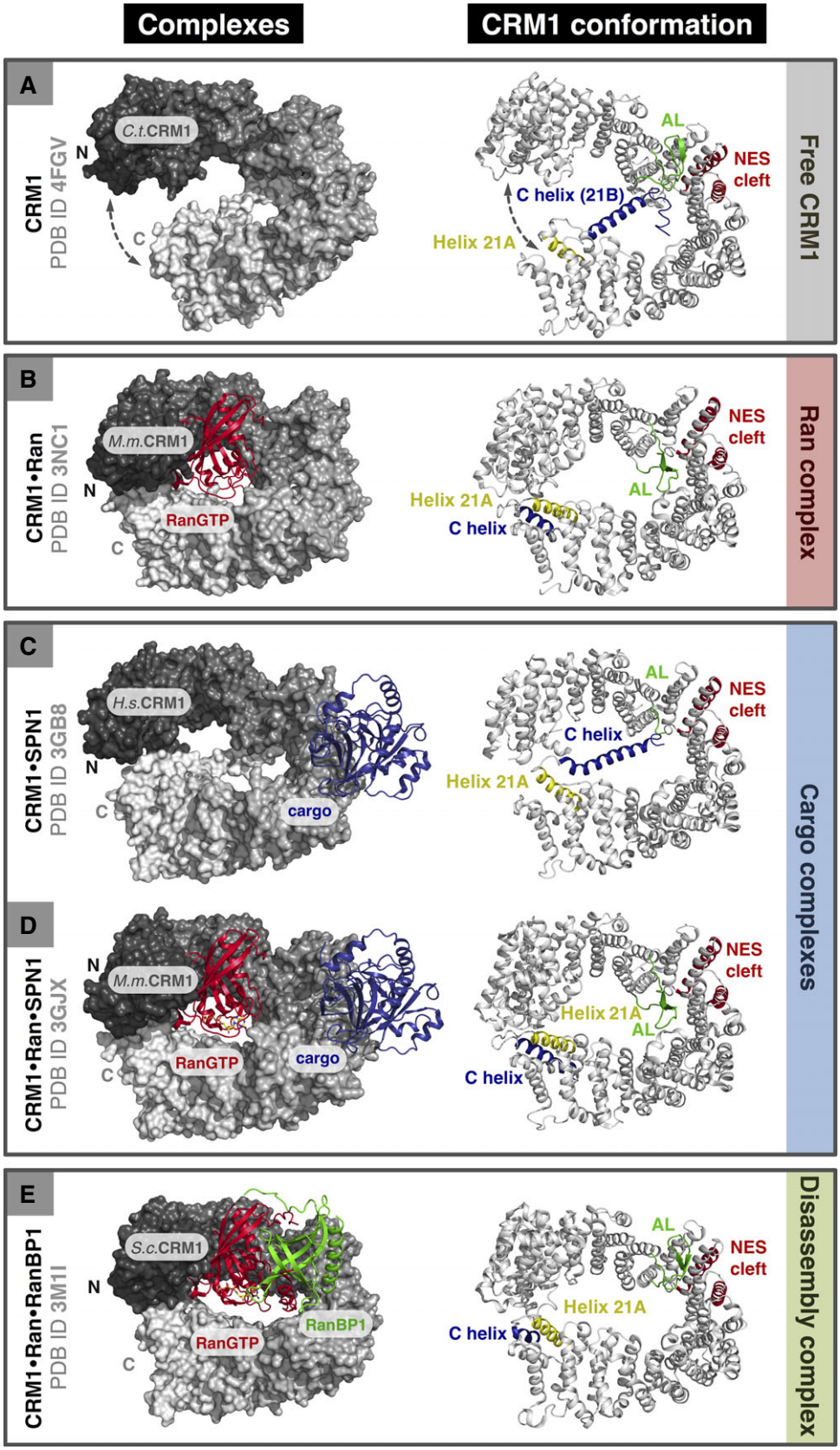


Fig. 2. HEAT-repeat architecture and overall structure of CRM1. (A) Structural organization of a HEAT repeat composed of two consecutive anti-parallel helices connected by a short linker loop. Commonly, the A-helix (red) is located at the outer, convex surface and the B-helix (yellow) lines the inner, concave area. HEAT repeats are characterized by pronounced intra-HEAT-repeat hydrophobic interactions. (B) CRM1 consists of an array of 21 consecutive HEAT repeats (H1–H21) forming an overall superhelical and flexible molecule. The acidic loop (green), NES cleft and the C-terminal helix with the adjacent C-terminal acidic tail are structural key features crucial for CRM1 function.

With respect to the overall structure of CRM1, the A-helices form the outer convex surface and may interact with phenylalanine-glycine-rich repeats of Nups during NPC passage. In contrast, the B-helices form the concave inner surface and interact with RanGTP [50] and, for most members of the importin β superfamily, with cargo (Fig. 2B).

Generally, importin β type nuclear transport receptors share a relatively low sequence homology of 10%–20%. The highest degree of sequence conservation between CRM1 and other importin β type transport receptors has been detected in the first three HEAT repeats. This N-terminal region has been termed the CRIME domain (CRM1-importin β etc.) [35,85] and is strictly required for the association with RanGTP (Figs 3 and 4). The CRIME domain binds to the switch II region of Ran, one of the two GTP-triggered switches common to small GTPases – hence nuclear transport receptors are able to sense the loading state of Ran [86,87]. Another important region involved in RanGTP binding is the acidic loop or HEAT9 loop (Fig. 4), a stretch of 26 residues of CRM1 forming a β-hairpin (Figs 2B and 3) [50]. In complex with cargo and/or RanGTP, the acidic loop adopts a seatbelt-like conformation, which locks Ran in a position intricately interacting with the CRIME domain of CRM1. In this conformation the acidic loop tip touches HEAT repeats 14 and 15 located on the opposing side of the CRM1 toroid (Fig. 3B,D) [50], thus increasing the rigidity of CRM1 in complexes [73]. In contrast, in the structure of CRM1 in complex with RanGTP and the Ran binding protein RanBP1 (Fig. 3E) the acidic loop is shifted from its seatbelt lock to a position that triggers RanGTP and cargo release [72]. An amazing and unique property of CRM1 is the atypical arrangement of HEAT repeat 21 and especially the orientation of helix 21B, for which two major conformations have been found (Fig. 3). In complex with SPN1 and RanGTP or RanGTP alone, the B-helix of HEAT repeat 21 is in a stacking arrangement to the other HEAT repeats (e.g. HEAT repeat 20), but in an inverted orientation facing the convex outer side of the toroid (Fig. 3B,D) [50,72]. In contrast, in the crystal structures of free CRM1 or bound to SPN1, the B-helix of HEAT repeat 21 spans the central channel of CRM1 and interacts with a region in the vicinity of the base of the acidic loop contacting the back side of the NES cleft (Fig. 3A,C) [51,74,75]. This again indicates the high structural flexibility of CRM1 and is further highlighted by the fact that not all N-terminally located HEAT repeats (CRIME domain) could be modeled in the Ran-free CRM1-SPN1 structure due the lack of electron density (Fig. 3C and Table 1). Moreover, the orientation of helix 21B has implications on the distance between the N- and C-terminal HEAT repeats. While in the parallel orientation (e.g. in the ternary CRM1-RanGTP-SPN1 complex) the N- and C-terminal regions form a tight and intricate interaction pattern (Fig. 3B,D), the conformation of free CRM1 lacks such interactions entirely (Figs 2B and 3A) [50,51,72,74,75]. An additional feature located C-terminally adjacent to the helix 21B is a stretch of acidic residues named C-terminal acidic tail (Fig. 2B). The electrostatic interaction of this stretch and basic resi-


Complexes
CRM1 conformation
A
CRM1
PDB ID 4FGV
C.t.CRM1
N
C
AL
NES cleft
C helix (21B)
Helix 21A
Free CRM1
B
CRM1•Ran
PDB ID 3NC1
M.m.CRM1
N
RanGTP
C
NES cleft
AL
Helix 21A
C helix
Ran complex
C
CRM1•SPN1
PDB ID 3GB8
H.s.CRM1
N
C
cargo
AL
NES cleft
C helix
Helix 21A
D
CRM1•Ran•SPN1
PDB ID 3GJX
N
M.m.CRM1
RanGTP
C
cargo
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Helix 21A
AL
C helix
Cargo complexes
E
CRM1•Ran•RanBP1
PDB ID 3M1I
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Disassembly complex

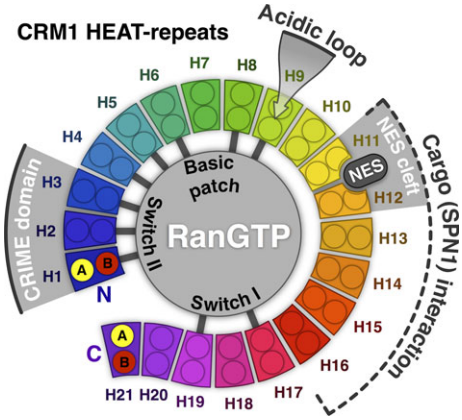

Fig. 4. Schematic representation of structural properties and cargo as well as RanGTP binding sites on CRM1. CRM1 HEAT repeats are represented by ring segments colored from dark blue (HEAT repeat 1, H1) to purple (HEAT repeat 21, H21). RanGTP is depicted as a gray circle with the location of the important switch regions I/II and the basic patch labeled. HEAT repeats interacting with RanGTP in crystal structures are connected to the respective part of RanGTP (gray line). The NES cleft is represented by a groove between HEAT repeats 11 and 12. HEAT repeats of binding sites for cargo, the location of the acidic loop and the CRIME domain are highlighted in gray.

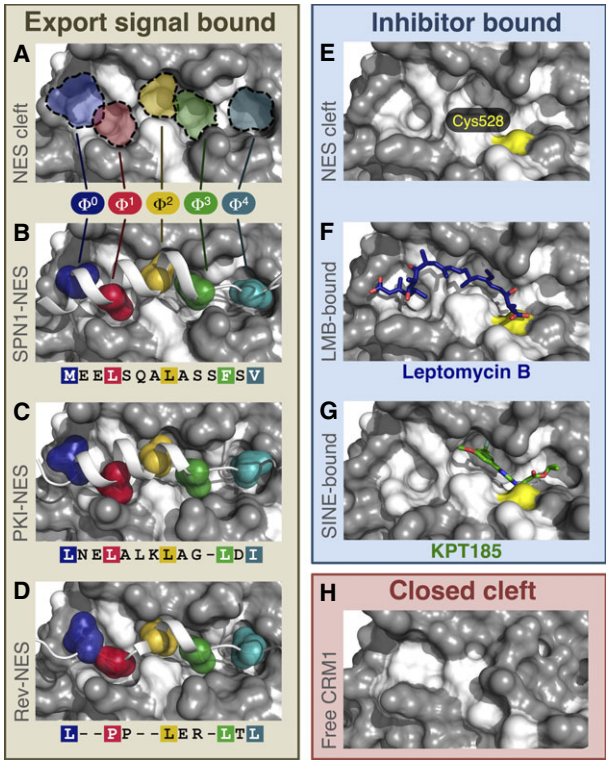

Fig. 5. Binding of nuclear export signals (NESs) and inhibitors to the NES cleft of CRM1. Detail view on the NES clefts of CRM1 bound to the SPN1-NES (A) and (B) (PDB ID 3GJX), PKI-NES (C) (PDB ID 3NC1) and Rev-NES (D) (PDB ID 3NBZ). The SPN1-NES has been removed in (A) for clarity to show the dimensions of the cleft and the respective Φ-pockets (blue, Φ^0; red, Φ^1; yellow, Φ^2; green, Φ^3; teal, Φ^4). Note that the key residues of all three NESs occupy identical Φ-pockets and differences in the Φ-spacing are compensated by a different arrangement of the NES-peptide main chain. Blocking of the NES cleft by CRM1-specific inhibitors LMB (E) and (F) (PDB ID 4HAT) as well as KPT185 (G) (PDB ID 4GMX). LMB has been removed in (E) to show the dimensions of the occupied NES cleft. In the closed NES cleft of free CRM1 (H) (PDB ID 4FGV) the Φ-pockets are inaccessible due to a 2.8 Å movement of helices 11A and 12A towards each other.

dues on the helix B of HEAT repeat 12 in proximity to the NES cleft have been shown to play a pivotal role in NES binding and release [88].

In contrast to other known exportins, CRM1 is a remarkable exception as it has been shown to bind cargoes on the convex surface of the superhelical toroid involving HEAT repeats 11–16 (Figs 3C,D and 4). Several crystal structures show how the cargo SPN1 interacts with CRM1 in a multipartite manner. SPN1 is a specific adapter for the import of uridine-rich small nuclear RNP (UsnRNP) core particles into nuclei after their cytoplasmic maturation step has been completed. After import, for the re-localization into the cytoplasm, SPN1 harbors an N-terminal NES (called SPN1-NES), which forms an amphipathic α-helix and contains five hydrophobic key residues (Φ-residues) for binding. These key residues dock into preformed hydrophobic pockets of the NES cleft of CRM1, which is located between the A-helices of the highly conserved HEAT repeats 11 and 12 (Figs 2B and 5B) [50,51,71]. Subsequent analysis of additional NESs of the cAMP-dependent protein kinase A inhibitor (PKI) and the HIV-1 protein Rev revealed that the identical pockets within the quite rigid NES cleft are used for binding of structurally distinct export signals (Fig. 5C,D). Because the five NES Φ-residues may

Fig. 3. Crystal structures of free CRM1 and CRM1-containing complexes. A surface representation of CRM1 (left panels) is shown gradient-colored from the N-terminus (dark gray) to the C-terminus (white) either alone or bound to various interaction partners (cartoon representation). Right panels show the conformation of the structural key features of CRM1 (cartoon representation) in the respective crystal structures of free CRM1 (A), a CRM1-RanGTP complex (B), a CRM1-cargo complex (C), a CRM1-RanGTP-cargo complex (D) and a disassembly complex (E). Proteins, originating organisms and key features are labeled and PDB IDs are given. AL, acidic loop; C helix, C-terminal helix. See main text for details.

exhibit different spacings in individual NESs, they require different arrangements of the NES-peptide chains to compensate for the differences in spacing (Fig. 5B,C,D) [71]. Moreover, the NES cleft of mammalian CRM1 contains a cysteine residue (Cys528 in human CRM1), which shows pronounced sensitivity to the CRM1-specific inhibitor LMB (Fig. 5E). Structural analysis revealed that LMB covalently modifies this cysteine in a Michael-type addition at the α,β-unsaturated δ-lactone moiety, and by spatial competition restricts binding of NESs to the binding cleft (Fig. 5F) [52,89,90]. Moreover, novel and small inhibitors of the SINE class (selective inhibitor of nuclear export) have been developed with the help of crystal structures which bind to the NES cleft and allow the (reversible) inhibition of CRM1-mediated export (Fig. 5G) [54,55].

In addition to the NES, other regions of SPN1 have been shown to play an important role in binding to the outer surface of CRM1. For example, the cap binding domain (CBD, amino acids 97–300) and C-terminal residues (amino acids 349–360) of SPN1 interact with A-helices of HEAT repeats 12–14 and 14–16, respectively (Figs 3C,D and 4) [18,50,51]. In general, other export cargoes might be recognized by a composite signal as well, e.g. the Rev protein mediating export of unspliced HIV-1 RNA [18,91]. Rev bears a second binding site interacting either as a compact entity like the SPN1-CBD or as a linear stretch with CRM1. This region strongly modulates the overall binding properties of Rev to CRM1 by a factor of 100 at sites corresponding to HEAT repeats 15 and 17 [91]. In fact, these HEAT repeats of CRM1 also interact with the C-terminal residues of SPN1 in the ternary CRM1-RanGTP-SPN1 export complex. Whether the regions surrounding the NES cleft of CRM1 are commonly used by other cargoes is still unclear, but the precise understanding of the binding mode of various cargoes would greatly help in the development of new potential CRM1 inhibitors for treatment of cancer. Interestingly, the surface of CRM1 reveals distinct charged and conserved patches in the vicinity of the NES cleft, which would enable cargoes with opposing surface charge to bind to other patches of CRM1 in addition to the common NES cleft interaction (Fig. 6, yellow contour).

The available structural information on CRM1 and its various export and disassembly complexes allowed the deduction of a detailed flowchart with the gross overall movements as well as the important interaction sites with Ran, cargo and disassembly factors (Ran-BPs) in the individual steps of a CRM1 transport cycle (Fig. 7).

Structural flexibility of CRM1 during the transport cycle

Assembly process

Free CRM1 in the nucleus serves as the starting point of the transport cycle. It has been shown that cargoes

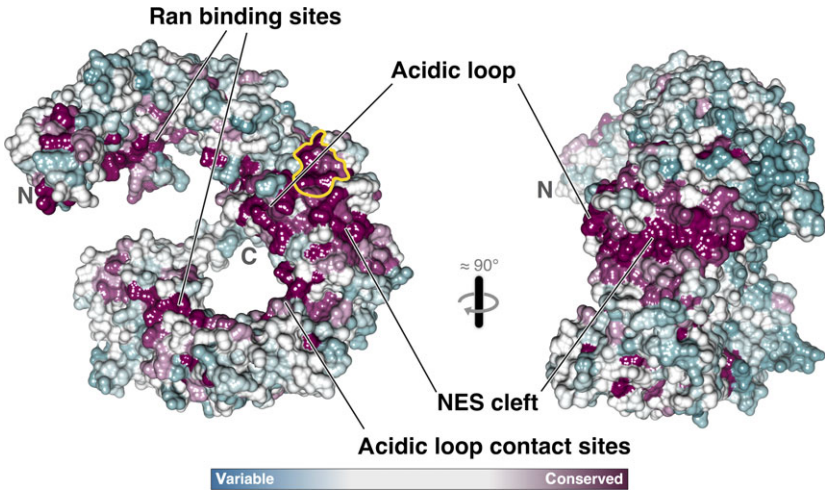

Fig. 6. Sequence conservation of CRM1 in the structural context of free CRM1 (PDB ID 4FGV). CRM1 is depicted in two orthogonal views and surface colored according to sequence conservation from invariant (deep purple) to variable (deep teal). The left view is identical to the orientation of CRM1 in Fig. 3A. Note that crucial structural key features like the acidic loop, the NES cleft as well as binding sites for RanGTP and contact sites of N- and C-terminal parts of CRM1 are well conserved. Conservation coloring is based on a multiple sequence alignment of 16 CRM1 orthologs from yeast to human. A conserved area in the vicinity of the acidic loop and the NES cleft is encircled by a yellow line and would be available for interaction with alternative composite export signals different from the one of SPN1.

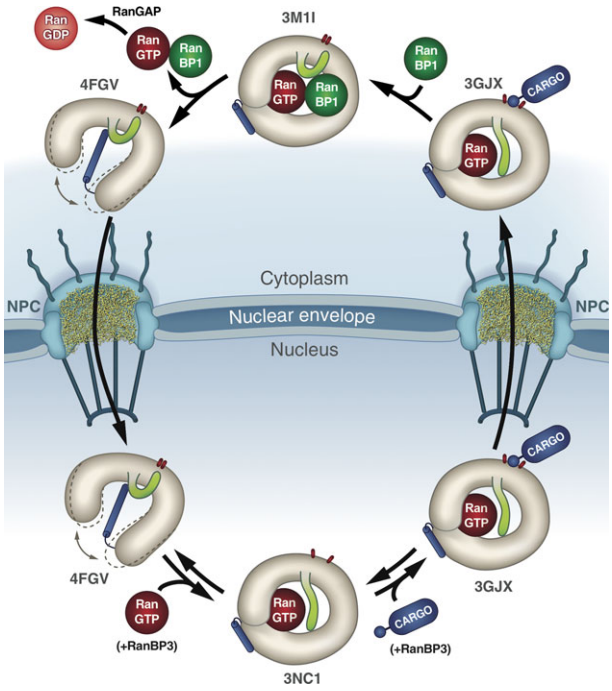

Fig. 7. Schematic overview of the individual steps and conformational changes during a CRM1 export cycle. The steps depicted highlight the different states of CRM1 (wheat) with respect to the overall shape as well as the positions of the acidic loop (light green), the C-terminal helix (HEAT helix 21B, blue) and the conformation of the NES cleft (red) during the transport cycle. The dashed line emanating from free CRM1 indicates its high structural flexibility. See main text for details.

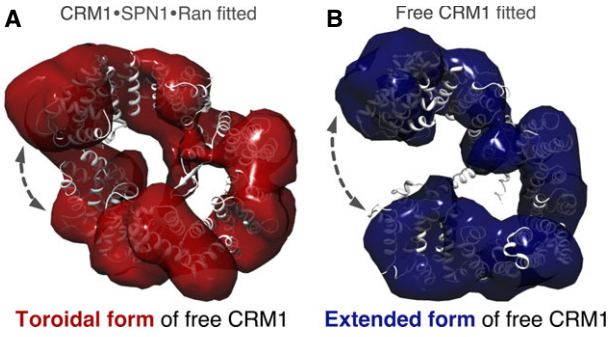

Fig. 8. Single-particle electron microscopy reconstructions of free *Chaetomium thermophilum* CRM1. CRM1 adopts toroidal (ring-like, red) (A) or extended (superhelical, blue) (B) conformations in a non-crystalline environment. The crystal structures of CRM1 in the ternary CRM1-RanGTP-SPN1 (PDB ID 3GJX) export complex and free CRM1 (PDB ID 4FGV) have been fitted into the toroidal and extended reconstruction, respectively.

in the absence of RanGTP have a relatively low affinity, which is increased strongly if RanGTP is present and *vice versa*. For the cargo SPN1, a binary CRM1-SPN1 complex has been described and crystallized rendering SPN1 an unusual cargo. This is further supported by the fact that in contrast to other cargoes the isolated NES of SPN1 has a rather low affinity for CRM1; however, this is more than compensated by the additional binding of the CBD and the C-terminal residues (Fig. 3D). Thus, at least for SPN1, two routes of export complex assembly (Ran or SPN1 first) could theoretically be envisioned. For each pathway crystal structures are available to provide structural information on a molecular level. However, since SPN1 is the exception rather than the rule, other cargoes are likely to associate stably only with a preformed CRM1-RanGTP complex (Fig. 7).

Structural investigations of free CRM1 (e.g. the cargo- and Ran-unbound form) have shown that it exists in two major conformations in equilibrium, namely an extended, superhelical conformation and a more compact, toroidal conformation (Fig. 8) [73,74]. Additionally, several crystal structures of the extended form have recently been determined at decent resolution and with reasonable model completeness (Table 1) [74,75] revealing an extended conformation of CRM1 entirely lacking interactions of the N- and C-terminal regions (Fig. 3A, left panel). Helix 21B is spanning the molecule reaching the opposing side of the superhelix (Fig. 3A, right panel) and touches the bases of HEAT repeats 9–12. The acidic loop in HEAT repeat 9 is oriented in a conformation binding to the B-helices of HEAT repeats 11 and 12 of CRM1, which form the NES cleft. Most probably these interactions stabilize the NES cleft in a closed conformation in free CRM1 thus precluding NES binding (Fig. 5H). Interestingly, the CRIME domain is quite distant to the other regions involved in RanGTP binding (Fig. 4), suggesting that RanGTP may freely access the N-terminal region of CRM1 in this state. Initial binding of RanGTP could trigger its progressive encircling by CRM1 (Fig. 3B). The helix 21B crossing the toroid in free CRM1 would clash with bound RanGTP (Fig. 3A). Thus, the displacement and rearrangement of this helix to the outside of the molecule (parallel orientation to helix 21A as in Fig. 3B) is a prerequisite for RanGTP binding. The large structural rearrangements of the acidic loop and the C-terminal helix of HEAT repeat 21 lead to a more compact structure with less superhelical pitch and tight interaction of N- and C-terminal regions mediated by a number of hydrogen bonds and salt bridges. Cooperativity is achieved by the movement of the acidic loop and its coupling to the NES cleft. After the release of the acidic loop from the back side of the NES cleft the equilibrium is shifted towards the open conformation,

which is prone for NES binding (Figs 3B and 5A). Consequently, the NES of a cargo can access the open NES cleft resulting in a locking of RanGTP by the acidic loop in the seatbelt conformation (Fig. 3B,D) and finally in an assembled functional export complex.

In the less extended, toroidal conformations that have been found to a varying extent in EM analysis (Fig. 8A) [73,74], the shape of CRM1 almost resembles that found in the CRM1-SPN1 complex (Fig. 3C). Interestingly, in this conformation helix 21B maintains the crossing position as observed in the extended, free conformation (Fig. 3C, right panel). However, the residues of the C-terminal acidic tail (amino acids 1063–1071 in human CRM1) are not defined in the electron density of the CRM1-SPN1 complex and thus are most probably disordered. This high congruence of the CRM1-SPN1 crystal structure to the EM model suggests that this conformation might be present already in solution and may be prone for binding to SPN1. This again shows the exceptional character of SPN1 as a CRM1 export cargo. In fact, SPN1 also seems to be an exceptional adapter for the nuclear import receptor importin β, as it exhibits different requirements for RanGTP-mediated release in comparison with other adapters like importin α [82,92]. In addition, for prototypical cargoes of CRM1, the nuclear RanGTP concentration *in vivo* might be significantly higher than the cargo concentration, and other auxiliary factors (e.g. RanBPs) are necessary for proper and efficient export complex formation. For example, RanBP3 is able to keep RanGTP bound to CRM1 for a prolonged time until an NES(-cargo) binds to the NES cleft and thus stabilizes the functional CRM1-RanGTP-cargo export complex [93].

Interestingly, the globular core domain of Ran in its GTP-bound state (excluding the C-terminal 36 residues) seems to be quite rigid in diverse crystal structures of transport receptor-RanGTP complexes (Table 2) [50,71,72,87,94,95]. This rigidity could be a requirement needed for the transduction of the cooperative effect from one side of CRM1 to the other binding site without direct interaction of RanGTP and cargo. Upon binding of RanGTP and/or auxiliary factors, the resulting structural changes on CRM1 are transduced via the key mediators, the acidic loop and the C-terminal helix to the still vacant cargo binding site, modulating its conformation to one prone for cargo binding (see above).

Table 2. The core domain of RanGTP (amino acids 1–170) is a rigid structure. RMSDs of known structures of RanGTP from different eukaryotes were compared with respect to structural differences.

PDB ID	Resolution (Å)	Comments	RanGTP $RMSD_{Chain}$ (Å)
3GJX	2.50	CRM1-SPN1-Ran	$Reference_C$
			0.13_F
3NC1	3.35	CRM1-Ran	0.44_C
2BKU	2.70	Importin β-Ran	0.48_A
			0.48_C
1IBR	2.30	Importin β-Ran	0.40_A
			0.40_C
1WA5	2.00	Cse1-importin α-Ran	0.42_A
3M1I	2.00	CRM1-Ran-RanBP1	0.44_A

To sum up the assembly process of an export complex with a prototypical cargo, biochemical studies show a RanBP3-mediated stabilization of the CRM1-RanGTP complex, hence promoting formation of a stable CRM1-RanGTP-cargo complex. This prediction is in line with structural data available so far: RanGTP and SPN1 as a cargo both stabilize CRM1 in its toroidal form and favor conformations of the acidic loop and the C-terminal helix that promote binding of the second molecule. The fact that a binary complex of CRM1 with the exceptional cargo SPN1 is stable enough to be purified and crystallized may indicate additional functions of CRM1 in the SPN1 cycle. *In vivo* localization studies show an enrichment of SPN1 in cajal bodies if CRM1 is blocked by LMB [96]. SPN1 in complex with the UsnRNPs travels to the cajal bodies, where the final assembly of UsnRNPs is thought to occur [97,98]. Once SPN1 is released from the UsnRNP, CRM1 may take over a chaperone function thus blocking rebinding of UsnRNPs to SPN1. In fact, it becomes more and more evident that nuclear transport receptors in general, besides their pure transport function, play pivotal roles in quality control (e.g. exportin-t for tRNA quality control [99]) or as chaperones (e.g. co-import of histone H1 by importin β and importin 7 [100]).

When the ternary CRM1-RanGTP-cargo complex is assembled, it traverses the NPC central channel to the cytoplasmic side. Current models predict interactions between phenylalanine-glycine-rich repeats in Nups and the convex, outer surface of nuclear transport receptors facilitating effective translocation through the NPC to its cytoplasmic side.

Disassembly process

After passage through the NPC, the disassembly of the ternary export complex is accelerated by RanBPs, aiding Ran-mediated GTP hydrolysis. One of them, RanBP2 (Nup358), is localized directly at the cytoplasmic side of the pore bound to the filaments emanating from the NPC whereas the other, RanBP1, is a soluble

protein [101–104]. These two RanBPs have been shown to increase the low intrinsic hydrolysis rate of Ran and affinity for RanGAP1. Sumoylated RanGAP1 is tethered to RanBP2 securing spatial vicinity and availability of both factors at a time to facilitate GTP hydrolysis [12,13]. Structural analysis of RanBP1-bound CRM1 complexes revealed that binding of RanBPs to the CRM1-RanGTP-cargo complex induces significant changes in the CRM1 overall conformation (Fig. 3E). In detail, the binding of RanBP1 fixes the acidic C-terminal DEDDDL sequence (amino acids 211-216) of Ran in a position on CRM1, which leads to the displacement of the acidic loop. Such displacement is achieved by substitution of acidic loop residues involved in the interaction with the switch I region of Ran and the adjacent CRM1 surface [72]. This re-localization of the acidic loop from RanGTP to the proximity of HEAT-repeat helices 11B and 12B on the inner side of the CRM1 toroid is thought to confer mechanical strain onto the HEAT repeats 11A and 12A leading to a constriction of the NES cleft and thus mediating release of the NES cargo [72,73].

Subsequently, the RanGTP-RanBP complex has to dissociate from CRM1 in order to interact with RanGAP1. Hence, Ran-bound GTP is hydrolyzed to GDP by RanGAP1 in the presence of RanBPs. RanGDP in turn exhibits a very low affinity towards CRM1 or CRM1-cargo complexes preventing formation of a new export complex.

Upon full export complex disassembly, keeping the NES cleft of free CRM1 in a closed conformation might be supported by the C-terminal helix (HEAT 21B) of CRM1. This helix has been found in two different conformations, one oriented in a parallel fashion to the other helices when RanGTP is present (Fig. 3B,D) [50,71,72], whereas it bridges the central hole of CRM1 in all others [51,74,75] (Fig. 3A,C). Further investigations led to the model that a C-terminal acidic tail of CRM1 following HEAT 21B forms electrostatic interactions with a basic patch on HEAT repeat 12B located in the vicinity of the acidic loop binding site and thus contributes to regulation of the NES cleft binding capabilities [88].

It remains an open question whether the movement of the helix 21B occurs on its own. MD simulations did not point towards independent opening of CRM1 enabling the repositioning. Nevertheless, this opening could require longer time scales than simulated by MD experiments so far. More likely additional factors are required to facilitate such re-localization; for example RanGAP1 binding to RanGTP-RanBP in the CRM1 complex may induce opening of CRM1. In fact, such opening would be a prerequisite for the relocalization of helix 21B to the final position observed in free CRM1.

Maintaining CRM1 in a free state for its traverse back into the nucleus is also possible by lowering the cytoplasmic pool of free cargo. For SPN1 it has been shown that importin β is able to displace it from the stable Ran-free SPN1-CRM1 complex *in vitro* [50]. This prevents rebinding to CRM1 and thus increases the probability that SPN1 binds its own cargo (UsnRNP core particles) by its CBD. In addition, for other cargoes it could be envisioned as well that cytoplasmic binding partners with a chaperoning function exist and are sufficient to prevent rebinding of the respective cargo to CRM1 independent of fixed positions for HEAT 21B and the acidic loop.

Cytoplasmic free CRM1 lacking any binding partners diffuses back into the nucleus for another round of export. Whether this occurs in a toroidal or extended overall conformation and whether the NES cleft remains closed during such passage due to its intrinsic properties or due to the binding of the acidic loop is still an open question.

Concluding remarks and future challenges

During the last decade, numerous crystal structures of CRM1 and CRM1 complexes have greatly facilitated the detailed understanding of CRM1 action and inhibition. CRM1 consists of 21 HEAT repeats and, depending on interacting partners, may adopt extended, superhelical (free form) or ring-shaped, toroidal conformations (in complexes). Bindings of RanGTP and cargo by CRM1 are cooperative events despite the fact that both molecules do not interact directly within an export complex. RanGTP is encircled by the B-helices of N-terminal HEAT repeats and additionally is kept in position by the acidic loop, while the cargo binds to A-helices of the central HEAT repeats located on the outer surface of CRM1. Key mediators of cooperativity are two regulatory regions of CRM1, the acidic loop and the C-terminal helix, which undergo large conformational rearrangements upon binding of a protein and thus transmit the binding information or facilitate a shift in the conformational equilibrium. Crucial hydrophobic Φ-residues of cargo-NESs neatly dock into the NES cleft of CRM1, which is formed between A-helices of HEAT repeats 11 and 12, and potent, mostly hydrophobic, inhibitors have been shown to block this cleft by spatial competition with cargoes. Moreover, crystal structures with the disassembly factor RanBP1 have shown how such stable export complexes can be resolved after NPC passage.

Although it may seem now that CRM1 action and inhibition is completely understood, there are still fundamental questions that remain to be solved. Future challenges are to unravel the molecular basis for the interaction of full-length prototypical cargoes like viral proteins or proto-oncogenes with CRM1 and the stabilization of such complexes by RanBP3. Moreover, it is currently unknown how more complex cargoes like ribosomal subunits, signal recognition particles or mRNAs are exported by CRM1. Although the first inhibitors of CRM1 are in clinical trials now, the structure-based development of highly effective CRM1 inhibitors with fewer side effects acting against various cancers is a formidable challenge. It remains a major and open question how the interaction of CRM1 with Nups is able to modulate the transition as well as the disassembly of CRM1 complexes. Finally, the structure–function understanding of alternative CRM1 functions, like in cell cycle control, is a major task and has just started.

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Author contributions

All authors contributed to structure analyses and writing the paper.

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