Focus Review

Ran-dependent nuclear export mediators: a structural perspective

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Nuclear export is an essential eukaryotic activity. It proceeds through nuclear pore complexes (NPCs) and is mediated by soluble receptors that shuttle between nucleus and cytoplasm. RanGTPase-dependent export mediators (exportins) constitute the largest class of these carriers and are functionally highly versatile. All of these exportins load their substrates in response to RanGTP binding in the nucleus and traverse NPCs as ternary RanGTP–exportin–cargo complexes to the cytoplasm, where GTP hydrolysis leads to export complex disassembly. The different exportins vary greatly in their substrate range. Recent structural studies of both protein- and RNA-specific exporters have illuminated how exportins bind their cargoes, how Ran triggers cargo loading and how export complexes are disassembled in the cytoplasm. Here, we review the current state of knowledge and highlight emerging principles as well as prevailing questions.

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Introduction

The nuclear envelope (NE) is certainly one of the most critical eukaryotic inventions. It encloses the cell nucleus, separates nuclear transcription and pre-mRNA splicing from cytoplasmic translation, and thereby allows eukaryotes to handle protein-coding genes containing introns (Alberts *et al*, 2007). This probably had a tremendous impact on eukaryotic evolution, because it aided, through exon shuffling, the appearance and diversification of multidomain proteins (Gilbert, 1978). In contemporary eukaryotes, the NE protects the genome (e.g., from reactive oxygen species originating

from mitochondria), and allows for a level of regulation that is unavailable to prokaryotes.

Such nucleocytoplasmic compartmentation requires a controlled exchange between the two compartments. Because nuclei lack protein synthesis, they import the required proteins from the cytoplasm (Bonner, 1975). Conversely, nuclei supply the cytoplasm with nuclear products such as newly assembled ribosomal subunits, tRNAs and mRNAs (Stevens and Swift, 1966). Within one second, nucleus and cytoplasm of a single proliferating mammalian cell actively interchange ≈ 1 million macromolecules with a total mass equivalent of $\approx 4 \times 10^{10}$ Da (Ribbeck and Görlich, 2001). Higher eukaryotes dedicate a transport system of ≈ 80 individual factors to accomplish this exchange (reviewed in Görlich and Kutay, 1999; Weis, 2003; Köhler and Hurt, 2007; Hetzer and Wentz, 2009; Stewart, 2010). These can be grouped into three functional categories, namely (i) nucleoporins as constituents of nuclear pore complexes (NPCs), (ii) shuttling adapter molecules and nuclear transport receptors (NTRs), (iii) components that feed metabolic energy into those transport processes, foremost including the players of the RanGTPase system.

NPCs are embedded into the double membrane of the NE (Watson, 1954) and thereby provide large aqueous channels (40–50 nm in diameter) that connect the cytoplasm with the nuclear interior. These channels are guarded by a sieve-like barrier that allows free passage of small molecules, but gets increasingly restrictive as the diameter of the diffusing species approaches a limit of ≈ 5 nm, which corresponds to a spherical protein of ≈ 30 kDa (Paine and Feldherr, 1972; Mohr *et al*, 2009). Larger objects can efficiently cross the barrier only when bound to appropriate NTRs (also called karyopherins/Kaps; Moore and Blobel, 1992; Adam and Adam, 1994; Görlich *et al*, 1994, 1995a,b; Chi *et al*, 1995; Imamoto *et al*, 1995).

NTRs continuously shuttle between nucleus and cytoplasm, bind their cargoes (directly or via adapter molecules) on one side of the NE, and release them on the other side, before they return to the original compartment to mediate another round of transport (for selected references see below). The permeability barrier of NPCs is formed by the so-called FG (phenylalanine/glycine) repeat domains of nucleoporins (Frey and Görlich, 2007; Patel *et al*, 2007). NTRs possess multiple binding sites for these FG repeats (Iovine *et al*, 1995; Radu *et al*, 1995; Bayliss *et al*, 1999, 2002; Bednenko *et al*, 2003; Isgro and Schulten, 2005) and interact with them in a way that allows the NTRs to overcome the size limit of the barrier and to cross NPCs within a few milliseconds (Kubitscheck *et al*, 2005; Yang and Musser, 2006; Frey and Görlich, 2007; Grünwald and Singer, 2010). NTRs typically accelerate NPC passage of cargo molecules by

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factors of 100 to >1000 (Ribbeck and Görlich, 2001) and can move objects of considerable size through the pores, examples being large ribosomal subunits (with a diameter of ≈ 25 nm), large (≈ 25 nm) gold particles or viruses with diameters of even nearly 40 nm (Feldherr *et al.*, 1984; Pante and Kann, 2002).

The superfamily of RanGTP-dependent receptors represents the largest NTR class and comprises 21 members in mammals and 14 in *Saccharomyces cerevisiae* (Görlich *et al.*, 1997; Fornerod *et al.*, 1997b). With respect to their activities, one can distinguish import mediators (importins), exportins and carriers that not only import one set of cargoes but also export others. All family members are sequence related to Importin β (Imp β). They bind RanGTP directly (Rexach and Blobel, 1995; Görlich *et al.*, 1996b, 1997; Fornerod *et al.*, 1997a) and use the metabolic energy supplied by the RanGTPase system as a driving force for directional transport (Moore and Blobel, 1993; Melchior *et al.*, 1993a). In response to RanGTP binding, they drastically change their affinity for cargo (Rexach and Blobel, 1995; Kutay *et al.*, 1997; Fornerod *et al.*, 1997a), exploiting the fact that the nuclear RanGTP concentration is ≥ 1000 -fold higher than the cytoplasmic RanGTP levels (Görlich *et al.*, 1996b, 2003; Izaurralde *et al.*, 1997; Richards *et al.*, 1997; Kalab *et al.*, 2002; Smith *et al.*, 2002).

Importins and exportins differ diametrically in the way they harness the RanGTP gradient: importins bind their cargo at a low RanGTP level (i.e., in the cytoplasm) and traverse the NPC as dimeric importin–cargo complexes. In the nucleus, RanGTP binding displaces the cargo and thereby renders import irreversible (Rexach and Blobel, 1995; Görlich *et al.*, 1996b). The resulting importin–RanGTP complex translocates back to the cytoplasm, where GTPase activation ultimately dislodges Ran from the receptor. Exportins operate in exactly the opposite manner, recruiting their cargo at high RanGTP levels in the nucleus (Kutay *et al.*, 1997; Fornerod *et al.*, 1997a). Here, cargo and RanGTP recruitment are coupled by positive cooperativity, that is, RanGTP increases ≥ 1000 -fold the affinity of the exportin for its cargo and, *vice versa*, the cargo stimulates RanGTP binding by the same factor. The ternary RanGTP–exportin–cargo complex crosses the NPC to the cytoplasm, where GTPase activation triggers disassembly of the export complex. The free exportin translocates back into the nucleus to mediate another round of export (Figure 1A).

Each importin- or exportin-mediated transport cycle removes one RanGTP molecule from the nucleus and releases it in its GDP-bound form into the cytoplasm. These NTRs, therefore, rely on additional components that refuel the RanGTP gradient: nuclear transport factor 2 (NTF2) retrieves RanGDP back to the nucleus (Ribbeck *et al.*, 1998; Smith *et al.*, 1998). Like all NTRs, NTF2 catalyses NPC passage of its cargo, but it is by sequence (Moore and Blobel, 1994) and structure (Bullock *et al.*, 1996) unrelated to Imp β . Nucleotide exchange (from GDP to GTP) on Ran is then catalysed by the guanine nucleotide exchange factor RCC1 (Bischoff and Ponstingl, 1991). RCC1 is chromatin bound (Ohtsubo *et al.*, 1989), and thus generates RanGTP exclusively in the nucleus. In contrast, the RanGTPase-activating protein RanGAP (Bischoff *et al.*, 1994) shows nuclear exclusion (Hopper *et al.*, 1990; Melchior *et al.*, 1993b), and thus depletes RanGTP selectively from the cytoplasm. RanGAP cannot

directly act on importin- or exportin-bound RanGTP. Instead, it has to cooperate with RanBP1 or the BP1-homologous Ran-binding domains (RanBDs) of RanBP2/Nup358 (Coutavas *et al.*, 1993; Yokoyama *et al.*, 1995) to activate the RanGTPase in those complexes (Bischoff and Görlich, 1997; Floer *et al.*, 1997; Kutay *et al.*, 1997; Lounsbury and Macara, 1997).

Interestingly, the general nuclear export pathway for mRNAs does not rely directly on RanGTP-binding exportins. Instead, the process is driven by the ATP-dependent RNA helicase Dbp5p (Snay-Hodge *et al.*, 1998), while NPC passage is mediated by the Mex67p–Mtr2p dimer (nomenclature for *S. cerevisiae*, the orthologous metazoan dimer is called TAP–p15 or NXF1–NXT1 complex; Kadowaki *et al.*, 1994; Segref *et al.*, 1997; Grüter *et al.*, 1998; Santos-Rosa *et al.*, 1998). The dimer is structurally related to NTF2 (Bullock *et al.*, 1996; Fribourg *et al.*, 2001). However, even mRNA export relies indirectly on the RanGTPase system, namely when importins retrieve mRNA export mediators and adapters back to the nucleus. In this article, we focus on the structural characterization of Ran-dependent exportins. For the related topics of mRNA export and nuclear protein import, we have to refer the reader to the excellent reviews written by others (Stewart, 2006, 2010; Cook *et al.*, 2007; Köhler and Hurt, 2007).

Functions of Ran-dependent nuclear export receptors

So far, eight RanGTPase-driven exporters have been identified in higher eukaryotes, while four export receptors are known in yeast (Table I). Exportins can vary widely in the number of different substrates they can handle. CAS (alias Exportin 2, also abbreviated as Exp2 or Xpo2 and called Cse1p in *S. cerevisiae*) for example, is fully dedicated to a single but highly abundant type of cargo. It recycles Importin α (Imp α , the Imp β -dependent import adapter for classic nuclear localization signals, NLSs) to the cytoplasm for a next round of import (Kutay *et al.*, 1997; Hood and Silver, 1998; Kunzler and Hurt, 1998; Solsbacher *et al.*, 1998). As expected from its essential role in NLS import, CAS is conserved and essential in all eukaryotes analysed so far.

Exportin 6 (Exp6, Xpo6) also serves an extremely narrow range of cargoes (Stüven *et al.*, 2003). It appears to recognize only actin directly and counteracts the slow (signal independent) leakage of this cytoskeletal protein into the nuclear compartment. A loss of Exp6 culminates in the appearance of nuclear actin paracrystals and is, at least in *Drosophila*, ultimately lethal. This illustrates an important principle, namely that the lack of a nuclear import signal is insufficient to keep proteins out of nuclei and that an exclusively cytoplasmic localization of a given protein is likely the result of steady nuclear export.

With respect to its cargo range, CRM1 (Exp1, Xpo1p) illustrates the other extreme—an exportin with a very large number of structurally unrelated cargoes (Fukuda *et al.*, 1997; Stade *et al.*, 1997; Wolff *et al.*, 1997; Fornerod *et al.*, 1997a). This broad specificity is possible because of an amazingly versatile cargo-binding site (see below), but also because CRM1 recognizes short peptide sequences, so-called leucine-rich nuclear export signals (NESs), that are readily accommodated into many proteins. In addition, adapter molecules

Figure 1 (A) Schematic overview of the nuclear export cycle. See main text for details. (B) The guanine nucleotide switch of Ran. Structure of Ran bound to GDP (left panel; Scheffzek *et al.*, 1995; Partridge and Schwartz, 2009; PDB-ID 3GJ0) or GTP (middle and right panels; Vetter *et al.*, 1999b; PDB-ID 1RRP). In the right panel, the structure of GTP-bound Ran is rotated to visualize the positively charged 'back' of Ran (with the twelve basic side chains represented by orange spheres). The central six-stranded β -sheet of Ran's G domain is shown in dark green; peripheral α -helices and loops are depicted in light green. Nucleotide binding is mainly accomplished by the G domain's loops (light green or colour coded), which contain several sequence motifs or residues that are highly conserved among guanine nucleotide-binding proteins (GNBPs; Vetter and Wittinghofer, 2001). The nucleotide is grey (sticks), the Mg^{2+} ion is shown as a sphere (magenta). When Ran switches between its nucleotide states, the so-called switch regions (switch I, human Ran^{30–47}; red; switch II, Ran^{65–80}; cyan; switch III, Ran^{177–216}; yellow) undergo drastic conformational rearrangements. There are also changes in the positioning and coordination of the Mg^{2+} ion. Upon nucleotide exchange (from GDP to GTP), switch I relocates. Helix α 1b melts completely and gets partially absorbed in α 1a, while β 2E dissolves. The conformational changes associated with switch II are more subtle, but functionally important: nucleotide switching positions Ran's catalytic glutamine side chain (not depicted) in closer proximity to the GTP γ -phosphate and thereby prepares Ran for GTPase activation (see main text). Switch III consists of a long linker, followed by an α -helix (α 6) and the highly acidic C-terminal DEDDDL motif. In RanGDP, the linker is close to the G domain and even contacts switch I, thus stabilizing the GDP state of Ran. Helix α 6 packs against the 'back' of Ran, while the DEDDDL motif probably contacts the so-called 'basic patch' (blue, Ran^{139–142}). Upon nucleotide exchange to GTP, switch I would clash into the switch III linker, triggering displacement of switch III. The resulting 'unlocking' of the Ran's back is crucial for transport receptor binding (see main text, Figure 2C). Switch III of RanGTP is shown in the RanBD-bound conformation; in the absence of a RanBD, it is disordered. (C) The RanGTP sensor of Importin β . *S. cerevisiae* Importin β (Kap95p) is shown in complex with RanGTP (Lee *et al.*, 2005; PDB-ID 2BKU). Imp β HEAT repeat helices are depicted as grey cylinders. HEAT repeats that interact with Ran are labelled and coloured in orange. In all contact maps, hydrophobic (distance ≤ 4 Å) and polar contacts (distance ≤ 3.8 Å) have been considered. HEAT 8 (in Ran-binding region 2) contains an acidic loop insertion that contacts Ran's basic back. Encircled numbers mark the distinct regions of Imp β 's RanGTP sensor. Ran is shown in tube representation, coloured as in (B).

recruit CRM1 to those cargoes that the exportin cannot bind directly (see Table I and below).

Exportin 7 (Mingot *et al.*, 2004) might also have a broad substrate specificity, however, so far only three cargoes have been characterized in detail. It functions together with the

adapter STRAD α to regulate the nucleocytoplasmic distribution of the kinase LKB1 (Dorfman and Macara, 2008) and counteracts the leakage of RhoGAP1 and 14-3-3 σ into nuclei (Mingot *et al.*, 2004). The cell thus employs multiple receptors for 'safe-guarding' the nucleus, which emphasizes that the

Table 1 Characterized vertebrate Importin β -like nuclear export mediators (exportins, abbreviated as 'Exp' or 'Xpo')

NTR	Cargoes (selection)	Adapter	Selected references
<i>Exportins</i>			
CRM1 (Exportin 1) Xpo1p/Kap124p	Leu-rich NES cargoes		Fischer <i>et al</i> (1995); Wen <i>et al</i> (1995); Fukuda <i>et al</i> (1997); Stade <i>et al</i> (1997); Fornerod <i>et al</i> (1997a)
	HIV genomic RNA	HIV Rev	Fischer <i>et al</i> (1995)
	m ⁷ G-capped UsnRNAs	PHAX + CBC	Izaurralde <i>et al</i> (1995); Ohno <i>et al</i> (2000)
	60S pre-ribosomal subunits	NMD3	Ho <i>et al</i> (2000); Thomas and Kutay (2003)
	Snurportin 1 (SPN1)		Paraskeva <i>et al</i> (1999)
CAS (Exportin 2) Cse1p/Kap109p	Importin α Srp1p/Kap60p		Kutay <i>et al</i> (1997); Solsbacher <i>et al</i> (1998)
Exp-t (Xpot) Los1p/Kap127p	tRNA		Kutay <i>et al</i> (1998); Arts <i>et al</i> (1998a); Hellmuth <i>et al</i> (1998)
Exportin 5 (Xpo5)	tRNA, eEF1A (via aa-tRNA)		Bohnsack <i>et al</i> (2002); Calado <i>et al</i> (2002)
	dsRNA-binding proteins (via dsRNA)		Brownawell and Macara (2002)
	Pre-miRNAs		Yi <i>et al</i> (2003); Bohnsack <i>et al</i> (2004); Lund <i>et al</i> (2004)
	60S pre-ribosomal subunits		Wild <i>et al</i> (2010)
Exportin 6	Actin–profilin complexes		Stüven <i>et al</i> (2003)
Exportin 7	p50RhoGAP, 14-3-3 σ		Mingot <i>et al</i> (2004)
<i>Bidirectional NTRs</i>			
Importin 13	<i>Import:</i> Mago-Y14, Ubc9, histone fold heterodimers		Mingot <i>et al</i> (2001); Kahle <i>et al</i> (2005); Walker <i>et al</i> (2009)
	<i>Export:</i> eIF1A		Mingot <i>et al</i> (2001)
Msn5p/Kap142p (orthologous to Exp5)	<i>Import:</i> RPA		Yoshida and Blobel (2001)
	<i>Export:</i> tRNA, dsRNA, phosphorylated Pho4p		Kaffman <i>et al</i> (1998); Shibata <i>et al</i> (2006)
Exportin 4	<i>Import:</i> Sox2, SRY		Gontan <i>et al</i> (2009)
	<i>Export:</i> eIF5A, Smad3		Lipowsky <i>et al</i> (2000); Kurisaki <i>et al</i> (2006)

S. cerevisiae NTRs that are functional orthologues of the vertebrate export receptors are named in blue. Only a selection of cargoes and references is listed. Adapters are proteins that link a cargo to an exportin and that are rapidly recycled back to the nucleus following export. See main text for more information.

exclusion of cytoplasmic components from the nucleus is a major task of exportins (see also below).

While the export mediators mentioned so far bind proteinaceous export determinants, Exportin-t and Exportin 5 recognize RNA-based export signals. Exportin-t (Exp-t, Xpot; Los1p in *S. cerevisiae*) appears fully specialized for nuclear export of tRNA, and serves a quality control function in that it preferentially exports matured tRNA molecules containing correctly processed 5' and 3' ends as well as appropriate nucleotide modifications (Hellmuth *et al*, 1998; Kutay *et al*, 1998; Arts *et al*, 1998a, b; Lipowsky *et al*, 1999). Exportin 5 (Exp5; Xpo5) is a second tRNA exporter, but apparently prefers a different tRNA spectrum than Exp-t (Bohnsack *et al*, 2002; Calado *et al*, 2002). It also exports ternary complexes of aminoacylated tRNA with eukaryotic elongation factor 1A (eEF1A), and thus depletes this translation elongation factor from the nuclear compartment. Exp5 binds and exports double-stranded (ds)RNA such as the adenoviral VA1 RNA and the human Y1 RNA (Gwizdek *et al*, 2003) as well as precursor microRNAs

(pre-miRNAs) of various lengths (Yi *et al*, 2003; Bohnsack *et al*, 2004; Lund *et al*, 2004). Further, Exp5 cooperates with CRM1 to export large ribosomal subunits from nuclei of higher eukaryotes (Wild *et al*, 2010).

So far, we know of three carriers that move distinct sets of cargoes in opposite directions (Table 1): Exportin 4 (Exp4) exports eIF5A and Smad3, but imports Sox-type transcription factors (Lipowsky *et al*, 2000; Kurisaki *et al*, 2006; Gontan *et al*, 2009), while Importin 13 (Imp13) carries the Mago-Y14 heterodimer (a component of the exon junction complex) into and eIF1A out of the nucleus (Mingot *et al*, 2001). Msn5p, the Exp5 orthologue in *S. cerevisiae*, can export dsRNA, tRNA (Shibata *et al*, 2006) as well as the phosphorylated transcription factor Pho4 (Kaffman *et al*, 1998) and transports replication protein A (RPA) into the nucleus (Yoshida and Blobel, 2001).

One might be tempted to assume that this impressive functional variety of exportins is reflected in different structural organizations. Surprisingly, however, Imp β -like NTRs share a very similar architecture.

Architecture of Imp β -like NTRs

The smallest architectural unit of Imp β -like NTRs is the so-called HEAT repeat (Andrade and Bork, 1995; Chook and Blobel, 1999; Cingolani *et al*, 1999; Vetter *et al*, 1999a), named after the prototypic proteins of this class: huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A and the lipid kinase TOR. One HEAT repeat comprises two α -helices (of 10–20 residues each) that are linked by a short intrarepeat loop. The α -helices (denoted A and B) pack to form a helical hairpin. About 20 of these hairpins stack with a slight clockwise twist, giving rise to right-handed solenoids (see below). In these superhelices, the A-helices face the convex back of the transport receptor while the B-helices constitute the ‘inner’ (concave) surface of the NTR. The intrarepeat and interrepeat contacts establish a continuous hydrophobic core. One can think of an NTR superhelix as of a tightly coiled spring in which each turn corresponds to one HEAT repeat (Stewart, 2003). Such repetitive structure is inherently flexible and this flexibility appears to contribute substantially to Ran-controlled cargo recognition and cargo release (Conti *et al*, 2006; see below).

Ran-controlled cargo loading by exportins

To operate as unidirectional cargo transporters, exportins must faithfully discriminate GDP-loaded from GTP-loaded Ran. At the same time, RanGTP binding needs to be coupled to cargo recruitment. How can such cooperativity be achieved? One can envision two distinct strategies. In the first, cooperativity arises because Ran and cargo contact each other in the export complex, that is, extra binding energy is released when both ligands bind simultaneously. In contrast to such a direct mechanism, Ran may facilitate cargo loading by triggering a conformational switch in the exportin, which activates the cargo-binding site—analogueous to the classic allosteric activation of haemoglobin by its ligand oxygen. As you will see below, both principles can be found among exportins, and they are not mutually exclusive: exportins that rely on direct Ran–cargo contacts also pass through different conformational states during their nuclear export cycles (see below).

Ran’s nucleotide switching is accompanied by dramatic conformational rearrangements of its G domain and its C-terminal extension (Figure 1B; Scheffzek *et al*, 1995; Chook and Blobel, 1999; Vetter *et al*, 1999a,b). Imp β -like NTRs do not directly sense the nucleotide in RanGTP, but instead probe those regions of Ran that differ most between the nucleotide states—the switch loops I and II. Ran’s C-terminus (‘switch III’) does not contribute to receptor binding, but has a pivotal role in the disassembly of NTR–RanGTP complexes (see below). NTRs also contact Ran at several ‘invariant’ loops and features of its ‘back’ (Figure 1B).

The general principles of RanGTP recognition by Imp β -like NTRs had first been revealed by the crystal structures of the Ran–Imp β and Ran–Transportin complexes (Chook and Blobel, 1999; Vetter *et al*, 1999a). However, as we discuss in the following sections, there are also decisive differences between Imp β and export receptors. In the case of Imp β , three distinct HEAT repeat regions contribute to Ran binding (Figure 1C): the receptor’s N-terminus (region 1) interacts with switch II as well as with the ‘back’ of Ran. Region 2 (which comprises an acidic insertion in HEAT 8) extends over

Ran’s back and shields, among others, the ‘basic patch’ (Figure 1B and C). The third region contacts switch I and those loops of Ran that hold the nucleotide’s guanine base. As compared with Imp β , all exportins characterized so far (CAS, Exp-t, Exp5, CRM1 and Imp13; see below) possess an additional (much more C-terminal) Ran-binding element (region 4; Figure 2; Matsuura and Stewart, 2004; Cook *et al*, 2009; Monecke *et al*, 2009; Okada *et al*, 2009; Bono *et al*, 2010). With the notable exception of Exp5, this C-terminal interface always contacts switch I of Ran. As a consequence, exportins clamp Ran from two sides—a topology that had already been suggested by Matsuura and Stewart (2004). In spite of this unifying theme, exportins vary in the way they engage their C-termini and acidic HEAT loop extensions to recruit Ran. These differences are seemingly minor; however, they reflect the shape diversity of NTR solenoids, their different cargo specificities as well as the way of how Ran and cargo recruitment are coupled.

CAS is highly specialized for Imp α recycling

The RanGTP–CAS–Imp α complex from *S. cerevisiae* is the first nuclear export complex whose structure had been solved (Matsuura and Stewart, 2004). As mentioned above, the exportin CAS is a key player in the classic nuclear import pathway mediated by Imp α (Kutay *et al*, 1997).

The import adapter Imp α (Görlich *et al*, 1994) grants NLS proteins access to the Imp β -dependent import pathway (Chi *et al*, 1995; Imamoto *et al*, 1995; Görlich *et al*, 1995a,b). It provides not only a recognition site for SV40-type and bipartite nucleoplasmin-type NLSs, but also an Imp β -binding (IBB) domain that confers Imp β -facilitated NPC passage (Weis *et al*, 1996; Görlich *et al*, 1996a). The IBB domain is highly positively charged and thus resembles NLSs—a similarity relevant for function (see below).

Cargo–Imp α –Imp β complexes form in the cytoplasm and translocate as an entity through NPCs (Görlich *et al*, 1996b). On the nuclear side, RanGTP binding to Imp β releases the Imp α –cargo complex (Rexach and Blobel, 1995; Görlich *et al*, 1995a,b; Lee *et al*, 2005). However, the spontaneous dissociation of an NLS cargo from Imp α is slow (Gilchrist *et al*, 2002). Nup50/Npap60 (Nup2p in *S. cerevisiae*) accelerates this step (Solsbacher *et al*, 2000; Gilchrist *et al*, 2002; Matsuura and Stewart, 2005), preparing Imp α for loading onto CAS. CAS then selectively binds and exports the NLS-free form of Imp α (Kutay *et al*, 1997); and thus ensures that only the import adapter, but not the just imported cargo, is recycled back to the cytoplasm (Kutay *et al*, 1997; Gilchrist and Rexach, 2003; Matsuura and Stewart, 2004; Sun *et al*, 2008). This asymmetry is only possible because Imp α can switch to an autoinhibited state, where the IBB domain folds back and occupies the NLS recognition site (Kobe, 1999). The IBB–Imp β interaction suppresses this autoinhibition during import. CAS, however, enforces the autoinhibited state by clamping the IBB domain onto the NLS-binding site (Matsuura and Stewart, 2004). Thus, CAS is not just an exporter, but also a compartment-specific antagonist of the NLS–Imp α interaction. The second function explains why a general exportin such as CRM1 could not replace CAS during evolution.

CAS holds both Ran and Imp α between its N-terminal and C-terminal arches (Matsuura and Stewart, 2004; Figures 2A, B and 3A) such that Ran and Imp α contact each other: Ran’s

Figure 2 Ligand binding by exportins. The exportin complexes are drawn to scale and in identical orientation with respect to Ran. HEAT repeat numbering is according to the original references. See main text for more information. (A, C, E, G) Exportin-RanGTP complexes are shown as described in Figure 1C. (B, D, F, H) Left: Exportins are depicted as in Figure 1C, but Ran has been omitted. Instead, the respective cargo molecules are shown in blue (cartoon or surface representation). HEAT repeats and interrepeat loops contacting the cargo are labelled and shown in orange. Cargo regions that are contacted by Ran in the export complex are coloured in green. Right: Structures are rotated to visualize details.

positively charged back is only weakly engaged in CAS binding (Figure 2A, Ran-binding interface 2). Instead, it binds a negatively charged C-terminal part of Imp α (Figure 2B), whose deletion has been reported to abolish

export complex formation (Herold *et al*, 1998). This export complex topology and the observation that both Ran and cargo stabilize the HEAT 19 loop (Figure 2A and B), show why stable Ran binding by CAS is strictly cargo dependent

Figure 3 (A–E) Comparison of the cytosolic and nuclear states of structurally characterized export mediators. The indicated transport receptors are shown in a surface representation, gradient-coloured from dark blue (N-terminus) to light blue (C-terminus). Left panels show the NTRs in their (Ran-free) cytosolic states, whereas right panels depict their nuclear (RanGTP-bound) forms. The orientations are the same with respect to HEATs 2–7. RanGTP is shown in green and the indicated transport cargoes are depicted in orange. The acidic loop of CRM1 is shown in magenta. Red arrows sketch the most apparent global conformational changes that occur upon RanGTP binding. See main text for details.

and, conversely, why cargo binding relies on Ran (Kutay *et al.*, 1997). In addition, cargo loading also requires Ran-driven conformational changes in the exportin: unliganded CAS is tightly closed by its C-terminal region clamping onto the N-terminal arch (Cook *et al.*, 2005; Figure 3A). This conformation clearly precludes cargo loading. However, when RanGTP intercalates between the two arches, it opens the transport receptor to a horseshoe-like structure, which can readily accommodate the export cargo (Figure 3A).

Ran-controlled tRNA recognition by Exp-t

The *Schizosaccharomyces pombe* RanGTP–Exp-t–tRNA export complex resembles that of CAS in topology and overall shape (Cook *et al.*, 2009; Figure 3A and B). As in the case of CAS, cooperative cargo and Ran binding is facilitated by a direct interaction between Ran's positively charged back and negatively charged features of the cargo (Figure 2C and D). Ran also promotes tRNA binding by inducing a conformational change in Exp-t. However, this conformational change differs markedly from that seen for CAS: cytoplasmic Exp-t is not closed, but instead rather elongated, with its N- and C-termini being distant in space. Ran stabilizes a more closed form of Exp-t that is compatible with tRNA binding (Cook *et al.*, 2009; Figure 3B). Thus, both CAS and Exp-t act as Ran-driven 'clamps', but the ways in which Ran regulates these clamps are diametrically opposite.

The structure of the Exp-t–export complex (Cook *et al.*, 2009) revealed how Exp-t can be a class-specific export receptor that discriminates tRNA from other highly structured RNAs. Exp-t acts as a 'molecular ruler' that probes the base of the tRNA's acceptor arm via its highly curved C-terminal arch on one end and the 5' and 3' termini via its N-terminal region on the other end of the tRNA (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999; Figure 2D). Apart from the Exp-t interaction with the tRNA's 3' end (see below), all major contacts involve the tRNA's phospho-ribose backbone. Thus, Exp-t selects its cargoes mainly by their unique shape and charge. The hypervariable loop and the anticodon loop, that is, the parts that differ most between tRNAs, are not part of the export signature.

After synthesis by RNA polymerase III, tRNAs undergo several nuclear maturation steps that include trimming of the 5' and 3' ends, post-transcriptional addition of the 3' CCA extension, base modifications and in some cases the removal of a short intron (reviewed in Phizicky and Hopper, 2010). Eventually, only mature and correctly folded tRNAs are exported to the cytoplasm. How can Exp-t accomplish such a role in nuclear tRNA quality control? The largely positively charged inner surface of Exp-t clamps onto the acceptor arm and the characteristically folded T Ψ C loop (Cook *et al.*, 2009; Figure 2D). Mutations that compromise this unique fold impede tRNA export (Tobian *et al.*, 1985).

Exp-t also recognizes the tRNA's termini. The 5' end sits in a pocket formed by HEAT repeats 6–8 and the 'basic patch' of Ran (Cook *et al.*, 2009). However, neither Ran nor Exp-t contacts the terminal 5' phosphate (Figure 2D). Instead, specificity is achieved solely by shape and charge complementarity. Indeed, a 5' leader would clash with the exportin, explaining as to why 5' extensions abolish Exp-t binding (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999). In contrast, Exp-t directly recognizes the 3' CCA phosphate groups and the CC bases via a groove within

its N-terminal arch (Cook *et al.*, 2009; Figure 2D). Consistent with that, a CCA deletion reduces the affinity for Exp-t. Re-addition of UUU leads to partial rescue, indicating that the correct length of the 3' end is more important than its actual sequence (Lipowsky *et al.*, 1999). The 3' end-binding site can only accommodate single-stranded RNA that is no longer than four bases (Cook *et al.*, 2009). Splicing of pre-tRNAs is not required for Exp-t binding (Arts *et al.*, 1998b; Lipowsky *et al.*, 1999). Rather, a Los1p/Exp-t deletion in *S. cerevisiae* causes a tRNA splicing defect (Hurt *et al.*, 1987), suggesting that the exportin not only binds unspliced tRNA precursors, but also channels them to the splicing endonuclease at the inner nuclear membrane, before nuclear export occurs. Aminoacylation is not a prerequisite for efficient Exp-t binding and export; however, the aminoacyl group of a charged tRNA would be well tolerated and could even be directly recognized by Exp-t (Cook *et al.*, 2009). Mature tRNAs (containing all base modifications) bind Exp-t 5–10 times more strongly than the unmodified forms (Lipowsky *et al.*, 1999). It is currently unclear how these modified nucleosides contribute to the export signature, but possibly they merely stabilize the overall tertiary structure of export-competent tRNA.

RNA export mediated by Exp5

The cell's second RNA-specific exportin, Exp5, is the export receptor for pre-microRNAs and as such greatly impacts those regulatory networks that rely on this type of RNA. Micro RNAs (miRNAs) are ≈ 22 nt single-stranded RNAs that have a central role in post-transcriptional gene regulation (reviewed by Kim *et al.*, 2009). Canonical miRNAs are generated from 500 to 3000 nt RNA polymerase II hairpin transcripts, which are 5' capped and poly-adenylated. These so-called primary miRNAs (pri-miRNAs) are cropped at the base of their hairpin stem by the nuclear RNase III-type protein Drosha, (a constituent of the Microprocessor complex), yielding the so-called precursor (pre-)miRNA—the cargo of Exp5 (Yi *et al.*, 2003; Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Zeng and Cullen, 2004). Only pre-miRNAs qualify for nuclear export; pri-miRNAs are not accepted by Exp5. A typical metazoan pre-miRNA has a characteristic 2 nt 3' overhang and comprises an ≈ 33 -bp stem that is closed by a loop. In the cytoplasm, pre-miRNAs are further processed to form the active miRNA ('guide strand'; Kim *et al.*, 2009).

The crystal structure of Exp5 bound to RanGTP and a pre-miRNA provides first structural insight into Exp5-mediated nuclear export (Okada *et al.*, 2009). As for Exp-t, charge complementarity between the exportin and the phospho-ribose backbone of the cargo allows Exp5 to recognize pre-miRNAs in a sequence-independent manner (Okada *et al.*, 2009). In the export complex, the two arches of Exp5 establish a U-shaped structure that sandwiches 16 bp of the double-stranded pre-miRNA stem (Figure 2F). Stems shorter than 14 bp do not bind Exp5 efficiently (Yi *et al.*, 2003; Zeng and Cullen, 2004). Like for CAS and Exp-t, Ran's positively charged back engages in cargo binding. Together with a long loop in HEAT 15, Ran locks the pre-miRNA in the export complex (Okada *et al.*, 2009). This orients the stem such that the hairpin loop (which is not resolved in the crystal structure) faces the open end of the 'U', whereas the 5' and 3' termini are placed in the sharp turn of the Exp5 solenoid (Figure 2F).

How can Exp5 distinguish mature pre-miRNAs from their precursors? Exp5 probes the pre-miRNA termini for shape complementarity (Okada *et al.*, 2009). RNA hairpins with long unpaired 5' and 3' ends do not efficiently bind to Exp5, whereas 3' extensions facilitate pre-miRNA recruitment (even when they are longer than two nucleotides; Zeng and Cullen, 2004). Indeed, unpaired termini would clash with the exportin, but HEATs 12–15 establish a positively charged tunnel to accommodate the 2-nt 3' extension of correctly processed RNAs (Figures 2F and 3C). This tunnel interacts extensively with the cargo and blocks the double-stranded portion of the stem from threading through the 'hole'. 5' Overhangs greatly impair Exp5 binding and hence Exp5-mediated export (Lund *et al.*, 2004; Zeng and Cullen, 2004). Modelling suggests that insertion of a 5' overhang (instead of a 3' extension) into the tunnel would cause the misplaced 3' end to clash with the exportin (Okada *et al.*, 2009). The structure also helps to explain how, during transport, pre-miRNAs are protected from nucleases (Yi *et al.*, 2003): in the tunnel, the RNA termini are completely shielded from exonucleases, while Exp5 and Ran protect all sides of the RNA stem from endonucleolytic attacks (Figure 3C; Okada *et al.*, 2009).

Exp5 can recognize structurally diverse RNAs

In contrast to Exp-t, Exp5 accepts dsRNAs whose stems vary in length. This appears possible because the 'U'-shaped Exp5 molecule cannot act as a 'molecular ruler' in cargo recognition (Figure 2F). In addition, Exp5 substrates may contain more bulky protrusions (Gwizdek *et al.*, 2003). These RNAs can be efficiently recruited, probably due to further opening of the Exp5 'U' (Okada *et al.*, 2009). Conformational flexibility in cargo binding has been reported for Imp β , which can assume different superhelical twists to recognize divergent import substrates (Cingolani *et al.*, 1999; Lee *et al.*, 2003; Conti *et al.*, 2006). Opening of Exp5 could indeed be possible without compromising the exportin's RanGTP sensor: the C-terminus of Exp5 binds Ran only weakly and does not interact with switch I. Instead, the N-terminal Ran-binding interface of Exp5 (interface 1; Figure 2E) contacts both switch regions of Ran. Furthermore, Exp5 completely lacks a central Ran-binding site (region 3; Figure 2E).

The RNA interface of Exp5 is almost twice as large as that of Exp-t. However, Exp5 binds its cargo mainly through many (individually weak) long-range interactions (Okada *et al.*, 2009). Such a binding mode should further facilitate flexible cargo recognition. How Exp5 recognizes tRNA is yet to be established. However, modelling suggests that tRNA would bind in an orientation inverse to that seen in the Exp-t complex (Okada *et al.*, 2009; Figure 2D and F). The structural basis for the different tRNA preferences of Exp-t and Exp5 (Bohnsack *et al.*, 2002; Calado *et al.*, 2002) is currently unclear.

As mentioned above, Exp5 can also export eEF1A via tRNA (Bohnsack *et al.*, 2002; Calado *et al.*, 2002). Exp5 and eEF1A recognize complementary features of their tRNA ligands, which is why eEF1A can access the Exp5 pathway. In fact, any dsRNA-binding protein could be an Exp5 cargo, provided that its RNA partner binds to Exp5 in a mode that still allows for piggybacking (Bohnsack *et al.*, 2002; Brownawell and Macara, 2002; Calado *et al.*, 2002). Double-stranded RNA

also appears to be the Exp5-dependent export signal of 60S pre-ribosomal particles, as competition experiments clearly suggest that the RNA-binding site of Exp5 serves for ribosome recruitment (Wild *et al.*, 2010). Still, interesting questions remain such as to which ribosomal RNA is contacted, which of its parts are recognized by Exp5 or indeed, how many Exp5 molecules are recruited to a 60S particle.

Diverse functions of CRM1

CRM1 transports a far larger number of different cargoes than any other exportin, and thereby impacts many central aspects of cellular physiology. First, it serves as an important node in numerous regulatory networks. For instance, it controls the nuclear activity of the cAMP-dependent protein kinase (protein kinase A, PKA) by depleting the PKA inhibitor (PKI)–PKA complex from the nucleus (Wen *et al.*, 1995; Fornerod *et al.*, 1997a). Here, PKI mediates the PKA–CRM1 interaction. Likewise, CRM1 keeps several transcription factors (such as NF-AT or YAP1; Kehlenbach *et al.*, 1998; Yan *et al.*, 1998) cytoplasmic, until adequate stimuli block the export and thereby trigger nuclear accumulation of the regulator.

Second, CRM1 is required for the infection cycles of many viruses. HIV-1, for example, hijacks the exportin for the export of its genomic RNA to the cytoplasm, where the next generation of viral particles is assembled (Malim *et al.*, 1989, 1991; Fischer *et al.*, 1995; Fornerod *et al.*, 1997a). The HIV-1 Rev protein is an essential adapter in this process: it binds to the unspliced viral RNA and recruits CRM1.

Third, CRM1 exports essential RNPs to the cytoplasm, examples being SRP, as well as 40S and the 60S pre-ribosomal subunits (Ciufo and Brown, 2000; Ho *et al.*, 2000; Moy and Silver, 2002). The 60S particles not only recruit CRM1 with the help of the export adapter NMD3 (Ho *et al.*, 2000; Gadal *et al.*, 2001; Thomas and Kutay, 2003), but successful NPC passage requires the assistance of additional export mediators, namely that of Exp5 in vertebrates (Wild *et al.*, 2010) or Mex67p, Mtr2p and Arx1p in yeast (Bradatsch *et al.*, 2007; Yao *et al.*, 2007; Hung *et al.*, 2008). This complex requirement probably reflects the extraordinary size of this cargo. CRM1 of higher eukaryotes is also essential for the maturation of spliceosomal U snRNPs. With the help of two adapters (the CBC complex and PHAX) CRM1 exports U snRNAs to the cytoplasm (Izaurrealde *et al.*, 1995; Ohno *et al.*, 2000). There, they assemble with Sm proteins and acquire a 2,2,7-trimethyl (m₃G) cap structure, before the adapter Snurportin 1 (SNP1) and Imp β reimport the matured U snRNPs into nucleus (Huber *et al.*, 1998).

Fourth, CRM1 actively maintains the exclusive cytoplasmic localization of RanBP1, of RanGAP, and of numerous translation factors (Richards *et al.*, 1996; Feng *et al.*, 1999; Maurer *et al.*, 2001; Bohnsack *et al.*, 2002). CRM1 thereby contributes (like Exp6, Exp7 and Exp5) to the identity of the nuclear compartment.

Finally, in analogy to CAS, CRM1 recycles SPN1, the already mentioned import adapter for U snRNPs, back to the cytoplasm (Paraskeva *et al.*, 1999).

As expected from such heavy duty, CRM1 is conserved and essential in all analysed eukaryotes. This also explains as to why certain prokaryotes (*Streptomyces* sp.) can kill predating or competing eukaryotic species with antibiotics such as

leptomycin B (Hamamoto *et al*, 1983) that covalently inactivate CRM1 (Kudo *et al*, 1998; Neville and Rosbash, 1999).

CRM1-dependent export signals

The best-known CRM1-dependent export determinants are the so-called nuclear export signals (NESs). Prototypic examples are the PKI NES (Wen *et al*, 1995) and the NES of the HIV Rev protein (Fischer *et al*, 1995). Since their discovery, NESs had commonly been referred to as 'leucine-rich' peptide stretches that harbour four hydrophobic key residues (denoted Φ^1 – Φ^4), following the consensus Φ^1 –(x)_{2–3}– Φ^2 –(x)_{2–3}– Φ^3 –x– Φ^4 (with 'x' preferentially being charged, polar or small amino acids; reviewed by Kutay and Güttler, 2005; see below). Recent structural insight into

CRM1–cargo recognition has, however, redefined this consensus (Güttler *et al*, 2010; see below).

Apart from these linear signals, CRM1 also recognizes more complex export signatures that include three-dimensional features of whole proteins or protein domains. SPN1 is the prototypic member of this class of cargoes (Paraskeva *et al*, 1999). It represents the most avidly binding cellular CRM1 cargo and was the first cargo to be successfully crystallized in complex with CRM1 (Dong *et al*, 2009; Monecke *et al*, 2009).

CRM1 binds cargo in a unique manner

In topology, CRM1 export complexes differ markedly from those of CAS, Exp-t and Exp5: CRM1 forms a toroid-like structure that almost entirely enwraps Ran (Monecke *et al*,

Figure 4 Binding of divergent NESs by CRM1. CRM1 is unique in that it is the only exportin that can achieve cargo specificity by recognizing very short linear and transplantable peptide signals that can be amazingly divergent—so-called nuclear export signals (NESs). The figure illustrates how CRM1 accomplishes multispecific NES recognition and shows the allowed sequence space of NES-active peptides. See main text for details. (A) Panels depict the surface of the CRM1 NES-binding site ('hydrophobic cleft'). Hydrophilic areas are shown in blue, hydrophobic regions in grey and Cys528 (the target residue of leptomycin B) in yellow. The indicated NES peptides (orange cartoon representation) bind to the hydrophobic cleft mainly via their Φ residues (Φ^0 ; Φ^1 – Φ^4 ; colour coded). In spite of their different Φ spacing patterns (see insets), the depicted NESs dock equivalent Φ side chains into virtually identical pockets within the hydrophobic cleft by assuming different backbone conformations. (B) NES sequence alignment based on the structures shown in (A). Colour coding is as in (A). (C) Consensus of NES peptides. Based on (A) and biochemical experiments (Güttler *et al*, 2010, and references therein), PKI-class and Rev-class NESs can be distinguished. At Φ^0 – Φ^2 , they differ drastically in the preferred Φ spacing and their amino-acid preferences at the Φ positions. In PKI-type NESs not all five Φ residues are essential for NES activity—each individual Φ residue is dispensable provided that the remaining four Φ positions are sufficiently strong. Judged from this multispecificity of the hydrophobic cleft, the number of NES-active peptides is enormous.

2009; Figures 2G and 3D). The inner surface of the CRM1 toroid is, therefore, not available for cargo binding. Indeed, CRM1 does not coil around its export substrates, but instead recruits cargo to the outside of its ring (Figures 2H and 3D; Dong *et al*, 2009; Monecke *et al*, 2009; Güttler *et al*, 2010). This is topologically reminiscent of the interaction of Imp β -like NTRs with FG repeats of nucleoporins (Bayliss *et al*, 2000).

Snurportin's export signature comprises three distinct areas: an N-terminal part, the central m³G cap-binding domain as well as a C-terminal stretch. By sequence, Snurportin's N-terminus resembles classic NESs and docks with five hydrophobic Φ residues into a hydrophobic cleft of CRM1 (Dong *et al*, 2009; Monecke *et al*, 2009; Figure 4A). The structures of classic NESs bound to the RanGTP-CRM1 complex proved that this hydrophobic cleft indeed serves as the generic NES-docking site of CRM1 (Güttler *et al*, 2010; see below), and that 'a consensus NES' contains not just four, but five Φ residues (Φ^0 , Φ^1 – Φ^4). The NES-docking site comprises five hydrophobic (Φ) pockets to accommodate all Φ residues. It displays an extreme degree of sequence conservation and harbours the cysteine residue (Cys528 in mammals) that is covalently modified by leptomycin B (Figure 4A).

How CRM1 binds highly divergent NESs

NESs are so divergent in sequence that it had been difficult to comprehend how a single receptor can recognize all of these signals. In particular, it was unclear how CRM1 copes with NESs that differ in the spacing of their Φ residues. This conundrum was solved by comparing the CRM1 complexes of three NESs that differ in Φ spacing, namely the SPN1 N-terminus, a derivative of the PKI NES and the HIV Rev NES (Figure 4A). One could have assumed that CRM1 employs alternative Φ pockets or adapts its Φ pockets to recognize these signals. However, this is not the case. Instead, the three NESs dock to the same five Φ pockets, and compensate deviating Φ spacings by different conformations of their backbones. As a result, equivalent Φ residues of different NESs occupy virtually identical positions in space (Güttler *et al*, 2010).

The CRM1-bound N-terminus of SPN1 is mostly α -helical (Figure 4A), whereas the PKI NES compensates its shorter Φ^2 – Φ^3 spacer by an earlier break in the α -helix. The Rev NES (LPPLERLTL) was previously assumed to bind to CRM1 via four Φ -leucines, following a Φ^1 xx Φ^2 xx Φ^3 x Φ^4 pattern (Fischer *et al*, 1995; Wen *et al*, 1995). While this spacing is correct for Φ^2 – Φ^4 , the structure revealed a strikingly different pattern for the more N-terminal region: the proposed Φ^1 -leucine docks into CRM1's Φ^0 pocket, whereas the following proline reaches into the Φ^1 pouch. The Rev NES, thus, exemplifies a Φ^0 Φ^1 Prox Φ^2 xx Φ^3 x Φ^4 signal (Figure 4A and B). Unlike SPN1 N-terminus and PKI NES, the Rev NES binds to CRM1 in an extended conformation and thereby compensates for its shorter Φ spacing. In fact, Rev-like and PKI-like NESs should be considered distinct NES classes: while proline at Φ^1 inactivates PKI-like NESs, the Rev Φ^1 -proline is absolutely required for detectable CRM1 binding. Moreover, Φ^0 appears to be essential for CRM1 binding of the Rev NES, but not for a PKI-type signal (Figure 4C; Güttler *et al*, 2010).

A unifying consensus for divergent NESs

The structures described above suggested a revised NES consensus (Figure 4C; Güttler *et al*, 2010) that accounts for

the sequence variations and the resulting affinity differences seen among export signals (see below). The consensus considers that CRM1 prefers NESs that dock with five (and not just four) Φ residues into the five hydrophobic pockets of the NES-binding site. It further accounts for the different side-chain preferences of the various Φ pockets, the preferred Φ spacer lengths, and the fact that acidic residues around Φ^0 enhance CRM1 binding (Figure 4C).

An NES that matches all those preferences is likely to bind CRM1 with extraordinary affinity (Güttler *et al*, 2010; see also Engelsma *et al*, 2004). However, such strong binding is counter-productive. The respective cargo will show significant binding even to the Ran-free form of CRM1, hence reenter nuclei together with the exportin, and also outcompete other CRM1 substrates (Engelsma *et al*, 2004). Indeed, such 'supraphysiological' NESs have been so far only been found in certain viruses that might take advantage from disabling the export machinery of their host (Engelsma *et al*, 2008; Atasheva *et al*, 2010). Cellular NESs, however, are normally downtuned by marked deviations from the consensus (Engelsma *et al*, 2004; Kutay and Gütinger, 2005; Güttler *et al*, 2010).

Many NESs, therefore, lack a hydrophobic Φ^0 position. Other Φ positions are dispensable too, provided that the other four Φ residues are sufficiently 'strong' to guarantee NES activity (Engelsma *et al*, 2008; Güttler *et al*, 2010). Such Φ -skipping yields NESs with seemingly 'exotic' Φ spacings that were previously difficult to comprehend (Kosugi *et al*, 2008). The greatest NES sequence variation, however, originates from the fact that a wide range of hydrophobic residues, such as Leu, Ile, Val, Met or Phe can yield active Φ residues (Bogerd *et al*, 1996; Zhang and Dayton, 1998; Kosugi *et al*, 2008; Güttler *et al*, 2010; Figure 4C).

In summary, it appears likely that any peptide can serve as an NES as long as its backbone conformation allows it to place a sufficient number of Φ side chains into the hydrophobic cleft. However, not all sequences that look like an NES do confer nuclear export activity in their original sequence context. In fact, NES-like sequences represent a rather frequent protein pattern that is also found in a very large number of proteins that are not exported by CRM1. In these cases, it is likely that the ' Φ residues' are buried in the hydrophobic core of a globular domain (Kadlec *et al*, 2004; la Cour *et al*, 2004; Hantschel *et al*, 2005). This is obviously a critical consideration for the prediction of active NESs. One should expect NESs not to occur in compactly folded domains, but only in disordered regions and/or near the termini of the candidate protein.

Mechanism of cooperative RanGTP and cargo binding to CRM1

CRM1 can operate as a cargo pump only if it binds cargo together with Ran in the nucleus and releases its cargo upon hydrolysis of the Ran-bound GTP. The exportin covers most of Ran's positively charged back (region 2 in Figure 2G) and thereby masks those Ran residues that engage in cargo contacts in the CAS, Exp-t and Exp5 complexes. A long β -hairpin insertion in HEAT 9 (the 'acidic loop', Figure 2G) spans the central hole of the CRM1 toroid, binds switch I of Ran and appears to 'lock' Ran close to CRM1's N- and C-terminal HEAT repeats. There are no direct RanGTP-cargo contacts, which begs the question of how Ran promotes cargo binding to

CRM1. Here, cooperativity must rely on an allosteric mechanism, that is, on a switch of CRM1 between a nuclear conformation with high affinity for cargo and Ran and a cytoplasmic form that lacks binding sites for the two ligands (Monecke *et al.*, 2009; Koyama and Matsuura, 2010; Fox *et al.*, 2011). It has been suggested that 'nuclear' CRM1 is 'spring-loaded' and that this tension is compensated for by the binding energies released at the CRM1–RanGTP and CRM1–cargo interfaces (Monecke *et al.*, 2009). For unliganded CRM1, only low-resolution structural information has been obtained so far by small angle X-ray scattering and electron microscopy (Fukuhara *et al.*, 2004; Petosa *et al.*, 2004; Fox *et al.*, 2011). However, the recent crystal structure of an export complex disassembly intermediate, stabilized by RanBP1 (Koyama and Matsuura, 2010; see below), provides proof for a RanGTP-driven allosteric mode of CRM1 cargo loading and unloading. Structural aspects of export complex disassembly will be discussed below.

What predestines CRM1 as a broad-range exportin?

CRM1 differs from the other exportins in two related aspects that we suggest being crucial for its ability to transport cargoes that vary greatly in size, shape and surface properties. First, CRM1 substrates dock to the exportin's outer convex surface (Figures 2H, 3D and 4A). Such topology should impose fewer

size and shape restrictions onto cargoes than the cargo wrapping by CAS, Exp-t or Exp5, and thereby predestine CRM1 for the transport of 'bulky goods' such as ribosomal subunits. Second, the assembly of CRM1 export complexes does not rely on obligatory Ran–cargo contacts. This allows recognition also of those substrates that cannot establish a Ran-binding interface. This appears particularly relevant for the short NES peptides, which can be easily 'appended' to an existing protein structure, and which therefore account for most of CRM1's cargo diversity. As NESs bury all their critical residues into the NES-binding site of the exportin, they simply have no potential to expose a Ran-binding site.

Cytoplasmic disassembly of nuclear export complexes

When export complexes reach the cytosolic face of the NPC, their disassembly requires the activation of Ran's GTPase. Given that cytoplasmic RanGAP stimulates the GTPase of free Ran by up to seven orders of magnitude (Bischoff *et al.*, 1994; Becker *et al.*, 1995), one might assume that such disassembly is straightforward to explain. However, the process is remarkably complicated. The primary reason is that exportins (like all other Imp β -like NTRs) protect RanGTP fully from RanGAP action (Floer and Blobel, 1996; Görlich *et al.*, 1996b; Kutay *et al.*, 1997, 1998; Askjaer *et al.*, 1998; Hellmuth *et al.*, 1998;

Figure 5 Cytoplasmic disassembly of RanGTP–CRM1–NES complexes. CRM1 helices are shown as dark grey cylinders. The hydrophobic cleft (HEATs 11 and 12; dark blue) and the acidic loop (magenta) are indicated. RanGTP is depicted in green; functionally important motifs are coloured as specified. GTP (black) and the Mg^{2+} ion (magenta) are shown for orientation. Numbers in circles denote those B-helices of CRM1 that contact the acidic loop (black circles) or RanBP1 (white circle). (A) Upper: RanGTP–CRM1 interaction in the mammalian RanGTP–CRM1–SPN1 complex (PDB-ID 3GJX; Monecke *et al.*, 2009). The acidic loop extends over HEAT repeats 10–15 and also interacts with Ran loops, including switch I. (Residues involved in this switch I interaction are shown as sticks.) Lower: The hydrophobic cleft of CRM1 is shown as in Figure 4A. The cleft is open and can thus interact with cargo. Cys528 is highlighted for orientation. (B) Upper: Overview of an export complex disassembly intermediate—the CRM1–RanGTP–RanBP1 complex from *S. cerevisiae* (PDB-ID 3M1I; Koyama and Matsuura, 2010). CRM1 and Ran are depicted as in (A), RanBP1 is shown in a surface representation (light grey). Note that RanBP1 and RanGTP interact in a tight molecular embrace with Ran's C-terminal switch III wrapping around RanBP1. RanBP1 also interacts with CRM1 HEAT 15 as well as the acidic loop. RanBP1 binding triggers major conformational changes in CRM1, mainly in the acidic loop, but also in the arrangement of HEAT repeats. Collectively, these changes close the hydrophobic cleft (lower panel) and thereby release the NES cargo. The loss of all major interactions between the acidic loop and Ran destabilizes the CRM1–RanGTP interaction. (C) Overlay of the RanBP1–RanGTP–RanGAP structure (Seewald *et al.*, 2002) with the CRM1–RanGTP–RanBP1 complex (B). The structures were aligned via their RanBP1–RanGTP modules. From this overlay, only the CRM1–RanGTP–RanBP1 complex and RanGAP (transparent surface representation, light blue) are shown. Note the severe clashes of RanGAP with CRM1.

Paraskeva *et al*, 1999). Indeed, the structure of the RanGAP–RanGTP–RanBP1 transition state complex (Seewald *et al*, 2002) revealed that RanGAP contacts a region on Ran that is inaccessible in NTR–RanGTP complexes (see also Figure 5C). Moreover, NTRs inhibit Ran’s intrinsic GTPase activity (Floer and Blobel, 1996; Görlich *et al*, 1996b) by forcing the catalytic glutamine residue in switch II (Q69 in human Ran) away from the γ -phosphate of GTP (Vetter *et al*, 1999a). Lastly, export complexes are kinetically so stable that one can expect the half times for their spontaneous dissociation to be in the order of an hour. The RanBDs of RanBP1 (Yrb1p in *S. cerevisiae*) or RanBP2/Nup358 (Coutavas *et al*, 1993; Wu *et al*, 1995; Yokoyama *et al*, 1995) relieve the block of Ran and allow RanGAP to trigger GTP hydrolysis and hence export complex disassembly (Bischoff and Görlich, 1997; Floer *et al*, 1997; Kutay *et al*, 1997; Lounsbury and Macara, 1997). The RanBDs are, therefore, essential players in the RanGTPase-driven transport cycles. It has been proposed that another major contribution to export complex disassembly comes from the exportins themselves, in that at least some of them are under tension when bound to Ran, and that this tension is relieved as soon as they ‘snap’ into their Ran-free low-energy states (Matsuura and Stewart, 2004; Monecke *et al*, 2009).

How do RanBDs contribute to export complex disassembly? In NTR–RanGTP complexes, Ran’s C-terminus appears to extend into solution (Chook and Blobel, 1999; Vetter *et al*, 1999a) and can therefore provide an initial ‘grip’ for a cytosolic RanBD. RanBDs bind Ran in a tight ‘molecular embrace’ (Vetter *et al*, 1999b): Ran’s C-terminal switch III region wraps around the RanBD, while the RanBD’s N-terminus clasps around Ran (with its acidic part in close vicinity to Ran’s ‘basic patch’; Figure 5B). In all export complexes analysed so far (with the exception of the RanGTP–Exp5–pre-miRNA complex), the Ran–RanBD embrace would cause the RanBD to clash with the exportin’s Ran interfaces 2 and 3 (Figure 2), as judged by the overlay of the Ran–RanBD structure (Vetter *et al*, 1999b) with exportin-bound Ran (Matsuura and Stewart, 2004; Cook *et al*, 2009; Monecke *et al*, 2009; Okada *et al*, 2009). This would readily explain as to why RanBDs can destabilize the NTR–RanGTP interaction. The extent to which RanBP1 contributes to export cargo release appears to depend on the architecture of the exportin as well as on its cargo (see below).

RanBP1 can disassemble the RanGTP–CAS–Imp α complex already in the absence of RanGAP and strip off Ran as a RanBP1–RanGTP dimer (Bischoff and Görlich, 1997). The RanBD would clash with nuclear (cargo-bound) CAS and might even interfere with Imp α binding to Ran’s basic back (Vetter *et al*, 1999b; Matsuura and Stewart, 2004). Together, this could already explain the instability of the tetrameric RanBP1–RanGTP–CAS–Imp α intermediate. The strict cooperativity of RanGTP and Imp α binding (Kutay *et al*, 1997) as well the relaxation of CAS to its cytosolic form (Matsuura and Stewart, 2004; Cook *et al*, 2005) should further contribute to export complex disassembly (see Figure 3A). Nevertheless, the tetrameric complex must represent the critical disassembly intermediate.

In the case of CRM1, the structure of one such disassembly intermediate has been solved in the form of the *S. cerevisiae* CRM1–RanGTP–RanBP1 complex (Figure 5B; Koyama and Matsuura, 2010). Even though RanBP1 does not release

RanGTP from CRM1, the hydrophobic cleft is closed and thus unavailable for NES binding (compare Figure 5A and B). This suggests that RanBP1 binding to a RanGTP–CRM1–NES complex triggers cargo release before GTP hydrolysis occurs (Koyama and Matsuura, 2010). Indeed, RanBP1 recruited to a RanGTP–CRM1–NES complex would clash with CRM1’s Ran-binding region 2, but also (together with switch III of Ran) collide with CRM1’s acidic loop as well as HEAT repeats 14 and 15. Consequently, the acidic loop assumes a highly compacted RanBP1-stabilized conformation, packing against the ‘back’ of CRM1’s NES-binding site (Koyama and Matsuura, 2010; Figure 5B). RanBP1 binding to CRM1 also triggers slight ‘opening’ of the exportin at HEAT repeats 12–19 (Koyama and Matsuura, 2010). Together, these rearrangements close the hydrophobic cleft and thereby cause CRM1 to release its cargo.

How can RanGAP act on the RanBP1–RanGTP–CRM1 complex? Given that the interaction interface of Ran with RanGAP is still buried in the disassembly intermediate (Figure 5B and C), the simplest possibility would be a spontaneous dissociation of the RanBP1–RanGTP dimer from the exportin. It is, however, also well possible that RanGAP breaks the Ran–CRM1 contacts, possibly with the help of RanGAP modules that do not act directly in GTPase activation. The C-terminus of CRM1 appears to stabilize the cytosolic state of CRM1 (Dong *et al*, 2009; Fox *et al*, 2011) and might thus also contribute to the release of RanBP1–RanGTP from CRM1. Once the RanGTP–RanBP1 complex is released, however, it represents the preferred RanGAP substrate (Bischoff *et al*, 1995). RanGAP does not supply a catalytic residue to Ran’s active site (as it is the case for Ras and Rho, for instance), but activates the GTPase by orienting Ran’s catalytic glutamine in switch II (see above) and by shielding the active site from solvent (see Seewald *et al*, 2002 and references therein). When Ran switches its conformation to the GDP state (Figure 1B), it becomes incompatible with exportin and RanBP1 binding, which renders export complex disassembly irreversible.

What are the future challenges?

In spite of illuminating structural insight into export complex disassembly, the question of how exportins release Ran and cargo in the cytoplasm is far from being solved. It seems likely that critical intermediates will be too unstable to be trapped in a crystal. In fact, one can expect a continuum of structures—from a first contact of a RanBD with exportin-bound Ran, to the eventual extrusion of the RanBP1–RanGTP dimer. Reconstructing such trajectory by molecular dynamics simulation appears to be a formidable, but highly interesting challenge.

A related problem is that RanBP1 and RanGAP not only represent the general disassembly factors for RanGTP–CRM1–cargo complexes (Kehlenbach *et al*, 1999; Paraskeva *et al*, 1999; Maurer *et al*, 2001), but also that CRM1 depletes RanBP1 and RanGAP from nuclei (Richards *et al*, 1996; Feng *et al*, 1999) in order to maintain the nucleocytoplasmic RanGTP gradient (Izaurralde *et al*, 1997). This poses the question as to how RanBP1 or RanGAP can be loaded onto the exportin without causing immediate disassembly of their own export complexes. In the case of RanBP1, a classic NES accounts for the exclusively cytoplasmic localization (Richards *et al*, 1996).

Thus, we should expect at least two RanBP1–CRM1–RanGTP complexes of markedly different configuration: The disassembly intermediate with a closed hydrophobic cleft (Koyama and Matsuura, 2010) and a still elusive RanBP1 export complex, where the NES is docked into the NES-binding site. How RanGAP is recognized as a cargo is also still unknown. So far, structural analysis revealed no NES motif that would be solvent exposed (Hillig *et al*, 1999). In fact, there are more CRM1 cargoes that do not carry obvious NESs, pointing to so far unexplored specificities of this exportin.

Two additional players with specific, but not fully understood, functions in CRM1-mediated export deserve further attention: the FG nucleoporin Nup214 and a factor called Yrb2p in yeast and RanBP3 in vertebrates. The conserved C-terminal Nup214 FG domain appears to display high affinity for just a single NTR species, namely the cargo- and RanGTP-bound form of CRM1 (Fornerod *et al*, 1997b; Askjaer *et al*, 1999; Kehlenbach *et al*, 1999; Hutten and Kehlenbach, 2006). Nup214 was, therefore, suggested to be a platform that captures CRM1 export complexes as they reach the cytosolic NPC side and to facilitate their disassembly (Askjaer *et al*, 1999; Kehlenbach *et al*, 1999). Indeed, it would be very interesting to see how this domain affects the reaction path of export complex disassembly.

The nuclear protein Yrb2p/RanBP3 contains FG repeats, a low affinity RanGTP-binding site and is required for efficient CRM1-mediated export (Noguchi *et al*, 1997, 1999; Mueller *et al*, 1998; Taura *et al*, 1998; Langer *et al*, 2011). It interacts specifically with the nuclear conformation of CRM1 and can, at certain stoichiometries, stimulate the recruitment of some (but not all) CRM1 cargoes to the exportin (Englmeier *et al*, 2001; Lindsay *et al*, 2001). Here, it would be very interesting to see the structural basis of these effects and to reconstruct the complete reaction path.

Another unresolved question concerns the bidirectional transporters Imp13, Msn5p and Exp4 (Kaffman *et al*, 1998; Lipowsky *et al*, 2000; Mingot *et al*, 2001; Yoshida and Blobel, 2001; Gontan *et al*, 2009). How can these act both as importin and exportin, moving distinct cargoes into opposite directions? One prerequisite is a binary NTR–RanGTP interaction of intermediate strength, allowing for positive cooperativity between RanGTP and export cargo binding, but also for efficient import cargo displacement by Ran. Indeed, the K_D s for the binary Exp4–RanGTP (30 nM; Lipowsky *et al*, 2000) or the Msn5p–RanGTP interactions (52 nM; Hahn and

Schlenstedt, 2011) are close to the geometric mean of the K_D s for the unidirectional Imp13 (0.5 nM; Bischoff and Görlich, 1997) and the unidirectional exportin CRM1 ($\approx 3 \mu\text{M}$; Paraskeva *et al*, 1999). The other prerequisite are adequate binding sites for import and export cargo.

Crystal structures of Imp13 bound to import cargo or Ran have been determined recently (Figure 3E; Bono *et al*, 2010; Grünwald and Bono, 2011). Imp13 coils around its import cargoes (the Mago–Y14 complex or Ubc9). In both cases, recruitment of the import substrate and RanGTP is mutually exclusive. This nicely explains as to how Imp13 can perform unidirectional cargo import. How Imp13 binds export cargoes remains to be determined. Bono *et al* (2010) suggested that the import cargo-binding site of Imp13 might also recruit proteins destined for export, provided that they do not interfere with RanGTP binding. Such binding would probably involve direct contacts between Ran and the export cargo. However, given that Imp13 and CRM1 are architecturally similar (Figure 3D and E), it is tempting to speculate that also in the case of Imp13, Ran can allosterically promote export cargo binding to the outer face of the ring. Interestingly, nuclear Imp13 assumes a more closed conformation as compared with the cytosolic form. ‘Compaction’ of the ring could indeed coincide with considerable shape changes in the outer Imp13 surface.

Finally, the structural basis of nuclear actin export needs to be explored. The corresponding export complex containing RanGTP, Exp6, actin and profilin (Stüven *et al*, 2003) might be of considerable medical importance: certain mutations in α -actin are known to cause intranuclear rod myopathies, where actin paracrystals accumulate inside nuclei (reviewed in Feng and Marston, 2009). We would not be surprised if at least some of those mutations exert their effect by disrupting the Exp6–actin interaction.

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Conflict of interest

The authors declare that they have no conflict of interest.

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