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Review Article

The CRM1 nuclear export protein in normal development and disease

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Abstract: CRM1 (Chromosomal Maintenance 1, also known as Exportin 1) is the major mammalian export protein that facilitates the transport of large macromolecules including RNA and protein across the nuclear membrane to the cytoplasm. The gene encoding CRM1 was originally identified in yeast as required to maintain higher order chromosome structure. In mammalian cells, CRM1 was found to bind several nuclear pore proteins hence its role in nuclear-cytosolic transport was discovered. In addition to nuclear-cytosolic transport, CRM1 also plays a role in centrosome duplication and spindle assembly, especially in response to DNA damage. The crystal structure of CRM1 suggests a complex protein that binds the Ran protein bound to GTP, allowing for a conformational change that facilitates binding to different cargo proteins through a nuclear export signal (NES). Included in the cadre of cargo are multiple tumor suppressor and oncoproteins as p53, BRCA1, Survivin, NPM, and APC, which function in the nucleus to regulate transcription or aid in chromosomal assembly and movement. An imbalance in the cytosolic level of these proteins has been observed in cancer cells, resulting in either inactivation (tumor suppressor) or an excess of anti-apoptotic activity (oncoprotein). Thus, the concept of inhibiting CRM1 has been explored as a potential therapeutic intervention. Indeed, inhibition of CRM1 by a variety of small molecules that interfere with cargo-NES binding results in cancer cell death. Whether all of these proteins together are responsible for this phenotype or whether specific proteins are required for this effect is unclear at this time.

Keywords: CRM1, nuclear pore complex, leptomycin B, p53, Survivin, APC, p27, NPM, BRCA1

The nuclear-pore complex of proteins

In the eukaryotic cell, diverse molecular functions such as DNA synthesis, RNA transcription and translation and protein processing, occur within distinct intracellular compartments. As a result, macromolecules that participate in these processes must be exchanged between these compartments. While small molecules (20-40 kD) can passively diffuse across compartments, larger molecules, including most proteins and RNAs, are transported by signal- and energy-dependent mechanisms [1].

The site of active transport between the nucleus and the cytosol occurs at a multi-protein complex called the nuclear pore complex (NPC, **Figure 1**) [1-3]. The NPC is composed of approximately 30 individual nucleoporins (Nups), with an approximate molecular mass of 125 MDa [4-6]. The core structure is symmetrical, consisting of a spoke-ring component surrounding the central-transporter. Short fiber-like structures extend from the NPC into the cytoplasm while basket-like structures extend into the nucleus [5, 7]. Four transmembrane Nups that anchor the NPC in the double lipid bilayer of the nuclear envelope have been described in vertebrate cells [8-12]. Approximately 15 different structural Nups form subcomplexes that participate in the formation of the NPC building blocks, including the nuclear basket and nuclear/cytoplasmic rings. Ten additional Nups are rich in phenylalanine-glycine repeats that mediate the interaction of the NPC with soluble nuclear transport receptors [13-15]. Each NPC rapidly transports hundreds of macromolecules bidirectionally [1]. A large number of proteins and ma-

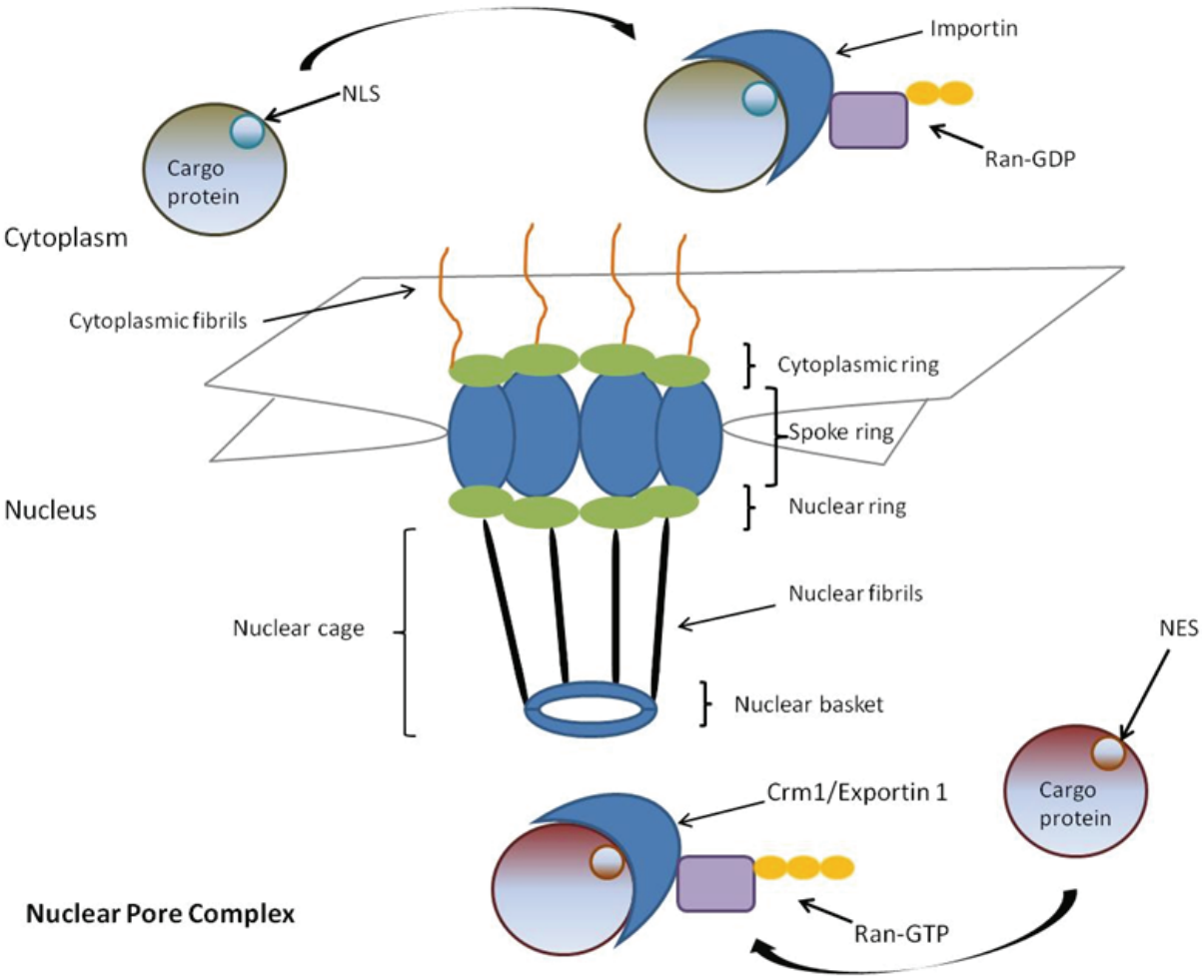

Figure 1. Model of the mammalian nuclear pore complex (NPC). The NPC is composed of nuclear pore proteins (Nups) surrounding a channel with a central transporter. The cytoplasmic fibrils and the nuclear basket assist in NES/NLS recognition and protein anchoring. Nuclear import is mediated by an importin bound to Ran-GDP. Nuclear export is mediated by the CRM1/Exportin 1 receptor bound to Ran-GTP.

ture snRNP particles are actively imported into the nucleus [1, 16], while export cargoes include proteins, mRNA, rRNA, tRNA, and snRNAs [17].

In most cases, energy-dependent, signal-mediated nuclear import and export involves nucleocytoplasmic shuttling receptors, which bind to their cargoes either directly or via adaptor molecules [1, 16, 18]. They are called importins or exportins, depending on the major direction of cargo transport, or collectively karyopherins [19]. After cargo molecules interact with their cognate receptors in the originating compartment, the transport complexes undergo stepwise translocation through the NPC to the destination compartment, whereupon the cargo dissociates from the receptor and the latter is recycled. Several different classes of transport signals have been described for nuclear import (nuclear localization signals, or NLS) and export (nuclear export signals, or NES). A "classical" NLS is composed of a short amino acid sequence enriched in basic amino acids that interacts with an import receptor consisting of an importin β and importin α heterodimer. A common type of NES is a short peptide segment enriched in leucine residues [20], which interacts directly with the nuclear export receptor CRM1/Exportin 1 [21-23].

The small GTPase Ran, which shuttles between the nucleus and the cytoplasm, plays a key role

in determining the directionality of nuclear transport [24, 25]. Many importin β nuclear transport receptors are RanGTP (Ran complexed with GTP) binding proteins [26]. The binding of RanGTP has distinct effects on import and export receptors. RanGTP dissociates cargo from nuclear import receptors [27, 28], while it promotes the association of cargo with nuclear export receptors [29-31]. Since the GTPase-activating protein for Ran (RanGAP) [32, 33] is sequestered in the cytoplasm and its guanine nucleotide exchange factor (RCC1) is restricted to the nucleus [34], most nuclear Ran is in the GTP-bound form, while most cytoplasmic Ran is in the GDP-bound form. Thus, the nucleocytoplasmic compartmentalization of Ran effectors in cells and the resulting asymmetric distribution of RanGTP are important for the loading and unloading of nuclear transport receptors in the nucleus.

The CRM1 mammalian export protein

The crm1 (chromosome region maintenance) gene was originally cloned in yeast after it was identified in a screen for genes essential for maintaining higher-order chromosome structure [23, 35]. Cold-sensitive (cs) crm1+ mutants of the fission yeast *Schizosaccharomyces pombe* resulted in deformed nuclear domains (abnormal chromosomal structures) at the restrictive temperature [35]. The crm1+ gene encodes a 115-kD protein that preferentially localizes to the nucleus and the nucleocytoplasmic junction and was originally found to interact with the AP-1 like transcription factor, pap1 [35]. Mutations in the fission yeast crm1 gene led to upregulation of pap1 [36] and functionally to a multidrug resistance phenotype, including resistance to caffeine and the anti-fungal agent leptomycin B [37-39]. A mechanism for these effects became apparent when the nuclear export function of CRM1 was discovered [40].

Human CRM1/Exportin 1 was originally cloned as a 112 kDa protein that interacted with at least two proteins associated with the human nuclear pore complex, the nucleoporins CAN/Nup214 and Nup88 [21, 41, 42]. Yeast two hybrid assays demonstrated interactions between CRM1 and several nucleoporins, as well as Rev and Ran proteins [43, 44]. Human CRM1 was subsequently identified as the human homolog of *S. cerevisiae* crm1 (47% identity 67% similarity) and to the *S. pombe* homologue (52% identity, 69% similarity). Further analysis revealed that the N-terminus of hCRM1 shared significant homology to that of importin β [42]. Cross-species comparison of the CRM1 protein sequence and structure indicates significant homology from yeast to humans (**Figure 2**).

The initial understanding of CRM1-mediated nuclear export mechanisms was greatly influenced by studies of the HIV-1 protein Rev [45]. The role of this protein in the viral life-cycle is to recognize and promote the export of unspliced and partially spliced viral RNAs. Rev and the protein kinase inhibitor (PKI) of cAMP-dependent protein kinase (cAPK) are the first proteins in which a NES was identified [46, 47]. Studies of the Rev NES led to independent identification of CRM1/Exportin 1. CRM1 was subsequently found to export a very broad range of substrates (4, 5, 7-11).

CRM1 crystal structure

The mouse and human CRM1 crystal structures were independently solved by two groups of investigators in a complex with the cargo protein snurportin 1 (SPN1). SPN1 is an import adapter protein that assists in transporting mature m3G-capped spliceosomal small nuclear ribonucleoproteins (snRNPs) into nuclei during their biogenesis [48]. To mediate import cycles, SPN1 is returned to the cytoplasm by CRM1 [49]. The crystal structure of the CRM1 complex confirmed prior protein sequence analyses and showed 20 HEAT repeats, each of which included two anti-parallel helices (A and B) lining the convex and concave sides of the protein (**Figure 3**) [50, 51]. In the complex, the CRM1 protein is bent to a distorted toroid structure, with HEAT 21 touching helices 2B and 5A, as well as the loop between HEATs 4 and 5. RanGTP is enclosed inside the toroid and stabilizes the ring closure through its multiple contacts. In contrast to other structural interactions with importin-β [52-54], the cargo protein SPN1 is not enveloped by CRM1 but rests outside of the CRM1 toroid. Further structural studies showed that CRM1 uses the same hydrophobic binding pocket for its interaction with different cargos, forcing NES peptide sequences to adapt their conformation to the rigid binding-site on CRM1 [55]. Depending on the spacing of key hydrophobic residues, the affinities of NES-containing cargos for their cognate receptor

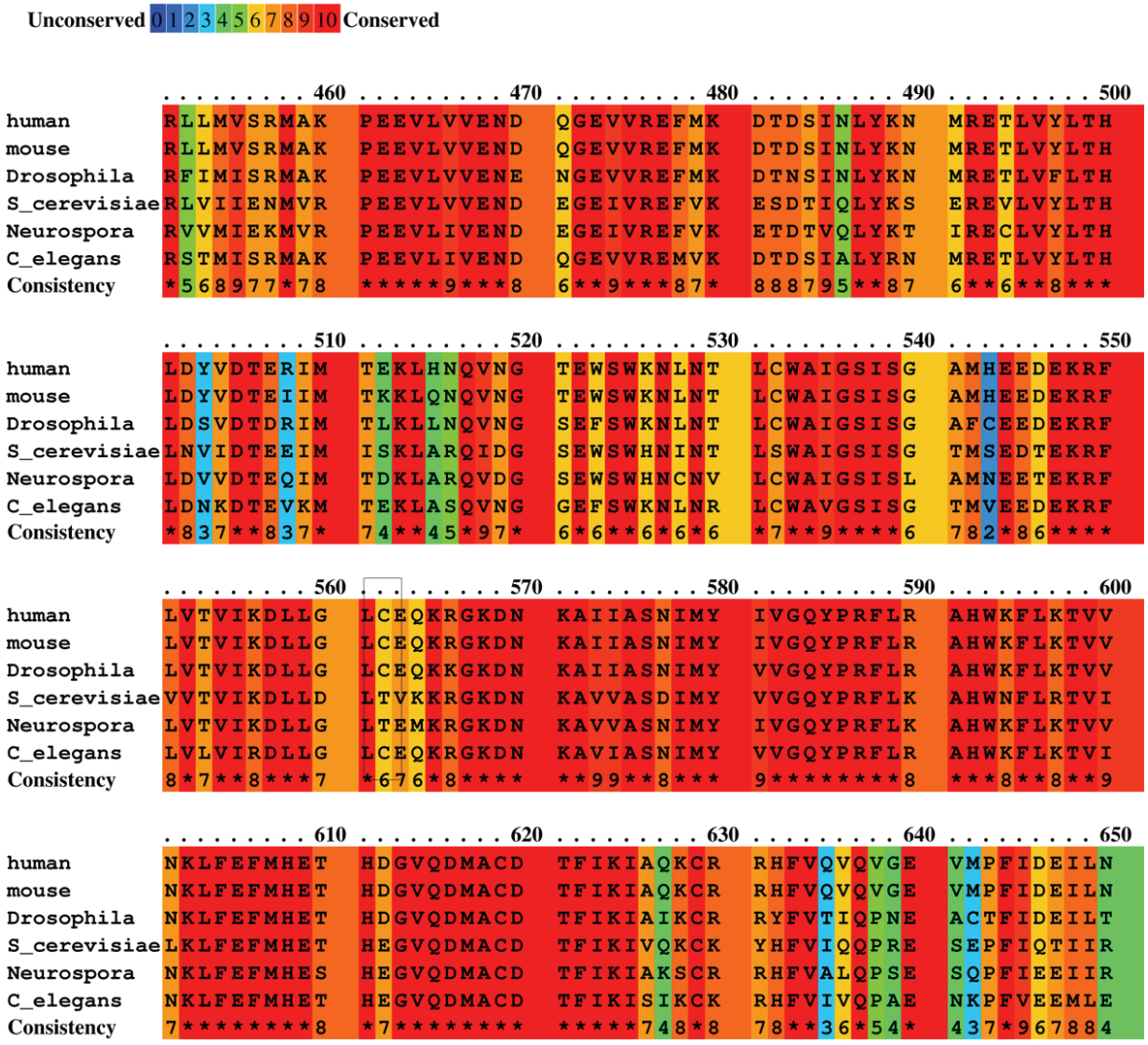

Figure 2. The central conserved domain (CCR) of CRM1 from six disparate species was aligned by ClustalW [149] and the results presented by PRALINE [150]. The part of the alignment containing the human Cys-528 residue that covalently binds to leptomycin B is marked by a box at 562 on the alignment number scale. Note that species that are leptomycin B resistant (here represented by *Saccharomyces cerevisiae* and *Neurospora crassa*) lack the cysteine at this position.

CRM1 can vary dramatically, with low-affinity cargos prevailing [56, 57]. Weak affinities seem to be important for efficient disassembly of export complexes on the cytoplasmic side of the NPC [58]. Furthermore, they prevent cargos from binding to CRM1 in the cytoplasm in the absence of RanGTP [56]. Another level of regulation of cargo binding to CRM1 appears to be the available concentration of CRM1 which is rate-limiting for export [59].

Expression and function of CRM1 in normal development

Regulation of the gastrula-neurula transition (GNT)

Developmental expression and function for CRM1 in higher eukaryotes has been explored genetically. In Xenopus, the CRM1-encoding mRNA is maternally expressed and is present throughout early development, which corre-

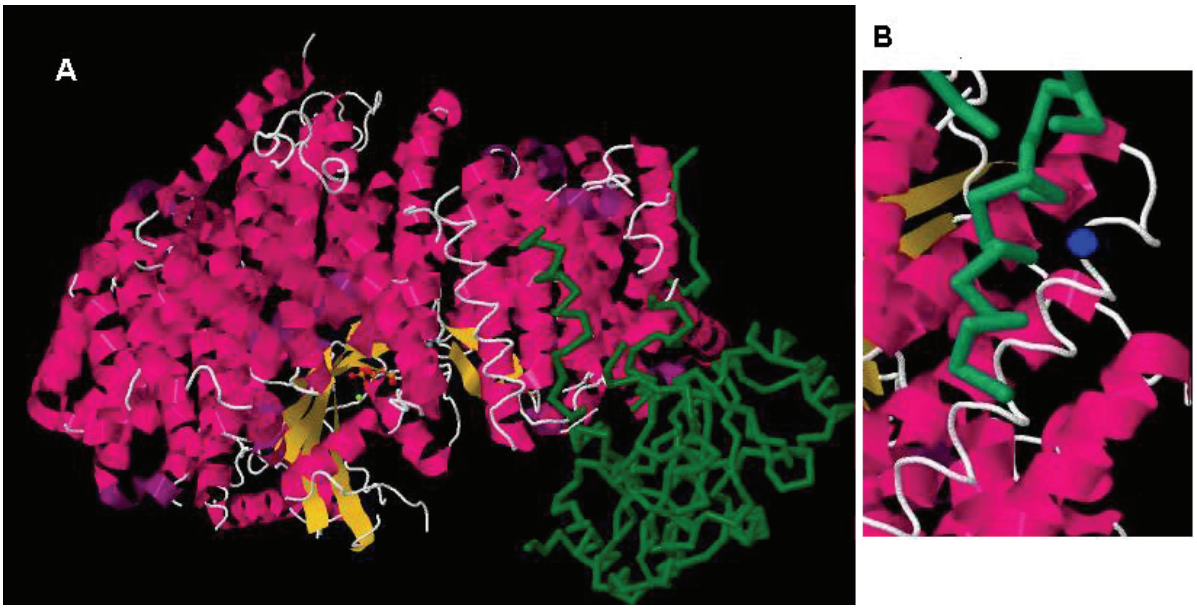

Figure 3. A. Crystal structure of CRM1, snurportin1 and RanGTP. The heat repeat anti-parallel alpha helices are in red, RanGTP is in yellow, and snurportin is in green. B. A portion of the crystal structure image of CRM1, snurportin1 and RanGTP highlighting the alpha helix containing Cys-528 and the hydrophobic cleft that binds a cargo protein's NES domain. The blue circle marks the position of the Cys-528 residue. The green backbone represents the amino terminal end of SNP1 which contains the NES domain of the protein. Image adapted from the RCSB PDB (www.pdb.org) of PDB ID 3GJX [50].

sponds with its constitutive protein expression [60]. XCRM1 localized within the nucleus, both before and during the gastrula stage. A specific nuclear membrane associated localization ('adult-type') CRM1 was observed beginning at the time of the neurula stage and CRM1 was unable to recognize the NES until this time period. In functional studies, XCRM1 RNA microinjections into stage 1 embryos showed arrested development before, during or just after the neurula stage, followed by death of the developing embryo, supporting a conclusion that unprogrammed activity of CRM1 greatly impairs normal Xenopus development and that the GNT is a critical period for its activity. Treatment of the embryos with the anti-fungal CRM1 inhibitor leptomycin B blocked development at the neurula stage, again suggesting that CRM1 is essential for procession through this stage and that inhibition of CRM1 activity does not affect normal development before this time [60]. Therefore, the intranuclear localization of CRM1 is under a developmentally controlled process and the GNT appears as an important period in the regulation of CRM1 activity during early Xenopus development.

Regulation of larval progression

In Drosophila, a screen for expressed sequences in chromosomal region 29C led to the isolation of a class of cDNAs encoding a polypeptide [61] with strong homology to the *S. pombe* crm1 protein [35]. Although originally named crm1 [62], the drosophila homolog was renamed embargoed (emb) to reflect the nuclear export defects observed in the emb mutant flies [61]. The emb transcript was ubiquitously expressed at all stages of embryonic development. Specific tissues in which expression was relatively high were brain, hind gut, and posterior spiracles shortly before dorsal closure and the ventral nerve cord, midgut, and somatic musculature shortly after dorsal closure [61]. In each case, emb expression levels increased when the tissue was mature, suggesting that it is required for the maintenance of these tissues rather than for their formation. Hemizygous emb flies showed an arrest in larval development at the transition from the second to third instar

stage [61].

Regulation of centrosome duplication and spindle assembly

The centrosome is the principal microtubule-organizing centre of mammalian cells, and functions to direct the assembly of a bipolar spindle during mitosis [63, 64]. Centrosome duplication is initiated at the G1/S boundary and is completed at S phase of the cell cycle, which coincides with DNA replication. Over the last decade several proteins that require CRM1 for their activity have been implicated in the regulation of centrosome duplication. These studies provide evidence of functions for CRM1 beyond nuclear-cytoplasmic shuttling.

Nucleophosmin

Nucleophosmin (NPM) is a centrosome-associated protein that is dependent on CRM1 for its nuclear-cytoplasmic shuttling during the cell cycle [65]. Mutation within the NES in NPM or disruption of CRM1 function by addition of leptomycin B, results in the dissociation of NPM from centrosomes and the initiation of premature centrosome duplication in addition to its effects on nuclear-cytoplasmic transport [65].

NPM is also a tumor suppressor protein in mice that likely serves the same functions in humans. This hypothesis is supported by the existance of NPM-associated chromosomal translocations as well as aberrant cytoplasmic protein expression in several hematologic malignancies [66-69]. The mechanism of NPM tumor suppressor function is likely to be controlled by its association with CRM1.

BRCA1

The breast and ovarian cancer susceptibility protein 1 (BRCA1) is a tumor suppressor protein encoded by a gene that when mutated is a risk factor for the development of breast and ovarian cancer [70-72]. BRCA1 regulates the DNA damage response and functions in the nucleus to stimulate DNA repair and at centrosomes to inhibit centrosome duplication after DNA damage [73-75]. At the centrosome, BRCA1 binds and ubiquitinates γ-tubulin which controls centrosome amplification and microtubule nucleation [76]. Inactivation of CRM1 impairs BRCA1 centrosome localization and mutation of the BRCA1 NES blocks BRCA1 regulation of centrosome amplification [77], suggesting that its interaction with CRM1 is essential for its function as a centrosome DNA damage checkpoint protein.

CRM1 can only bind to the undimerized form of BRCA1, as the NES is a core part of the binding domain used in heterodimerization of BRCA1 with its partner protein BARD1. Both BRCA1 and BARD1 NES become masked upon heterodimer formation [78]. A hypothesis based on these findings suggests that the ability of CRM1 to drive BRCA1, BARD1, and several BRCA1-BARD1 substrates, to the centrosome increases the proteins' proximity to one another, driving BRCA1-BARD1 dimer formation to catalyze ubiquitination of downstream substrates that are required to regulate centrosome amplification during the DNA damage response [77].

CRM1-mediated export of cancer proteins

An increasingly large number of cancer-associated proteins that shuttle into and out of the cell nucleus, as tumor suppressor and oncogenic proteins, require CRM1 for their nuclear exit (recently reviewed in [79]). Interestingly, mutations and/or dysregulation of such proteins in cancer cells, including BRCA1, p53, p27, and APC can lead to an aberrant high level of expression within the cytosol which disables them from performing their normal functions within the nucleus. We review here four proteins with distinct roles in cancer cells that are dependent on CRM1-mediated nuclear export.

Survivin

Survivin is a bifunctional protein involved with regulating cell division when expressed in the nucleus and controlling apoptotic pathways when expressed in the cytoplasm [80, 81]. The smallest member (16.5kDa) of the inhibitor-of-apoptosis protein family (IAP), survivin is believed to exist predominantly as a homodimer [82, 83]. Survivin export from the nucleus to the cytoplasm is mediated by the CRM1/Ran-GTP axis [84-86]. Survivin contains two amino acid sequences involved with its nuclear export: the first is the leucine-rich NES located in the linker region between the N-terminus and BIR domains of the survivin homodimer and the second is a non-classical sequence in the C-terminus [85, 87]. The central NES is partially

masked by the homodimer interface and is therefore primarily active when survivin is a monomer. The monomeric configuration of survivin can be induced by HDAC6-mediated deacetylation at lysine 129 [88, 89]. Upon interruption of the CRM1/Ran-GTP axis via disruption of the Ran-GTP gradient or by leptomycin B, survivin localizes to the nucleus, a process that thus far is thought to occur by passive diffusion as no classical NLS exists within the protein.

In the nucleus, survivin is primarily associated with the chromosome passenger complex (CPC), a core complex of proteins including Borealin, Aurora B kinase, INCENP and survivin, involved in ensuring the correct attachment between the centromere and the mitotic spindle, among other related checkpoint regulation pathways of mitosis [90, 91]. CRM1 is a necessary effector in co-localizing the CPC with the centromere during G2/M phase of the cell cycle by interacting with survivin [84, 85]. While not essential in CPC function or anchoring, CRM1 is required as a transient transporter of the CPC to the centromere [84, 92]. Aurora B kinase also accesses the CRM1-dependent nuclear export pathway for nucleocytoplasmic localization in a manner very similar to the survivin-CRM1 interaction - by interacting with the non-catalytic N-terminal domain of Aurora B [93, 94]. Mutational studies demonstrated that CRM1 interacts with the CPC through the NES of survivin [84]. The nuclear survivin-CRM1 interaction, independently of the CPC, may also negatively regulate transcription factors, such as oncogenic STAT3 [88].

In the cytoplasm, survivin exercises its cytoprotective function by actively inhibiting apoptotic pathways, to prolong cell life [95, 96]. Many studies have shown that survivin is both upregulated and localized in the cytoplasm of cancer cells [97, 98]; its nuclear export required for its anti-apoptotic and tumor-promoting function [99, 100]. Experiments disrupting the survivin NES sequence or CRM1 function in cancer cells result in nuclear localization of survivin, which increases the susceptibility of these cells to conventional chemotherapy and radiation treatment. The withdrawal of survivin by the collapse of the CRM1/Ran GTP axis followed by ubiquitin -proteasome degradation indicates an active regulatory mechanism that promotes cell death progression [100].

A variety of human splice variants for survivin exist, though not all isoforms are uniformly present or unambiguous [101, 102]. The NES is present in the canonical survivin, survivin2B, and survivin3B, but not in survivinDEx3 or survivin2α [103]. Though currently still ill-defined in vivo, expression of these isoforms has been suggested to correlate with certain disease models and clinical outcomes for cancer patients.

p27^{KIP1}

The Cdk inhibitor p27 is an important regulator of G1 progression in normal cells. It is highly expressed in G0, where it binds tightly and inhibits cyclin E-Cdk 2 [104-106]. In mid-G1, p27 also plays a role in the assembly and nuclear import of D-type cyclin-Cdk complexes [107]. p27 levels are regulated by translational controls and by proteolysis, and decrease as cells progress from G1 to S phase [108, 109]. Detectable p27 is exclusively nuclear in G0 and early G1, with a transient appearance in both the nucleus and cytoplasm as cells progress through G1, before its disappearance in late S phase [110]. The dramatic increase in p27-CRM1 binding during G1 progression and the transient appearance of cytosolic p27 at the G1/S transition suggested a link between nuclear export of p27 and its degradation [110]. The timing of the cellular p27-CRM1 interaction and the observation that p27 is exported more rapidly from G1 nuclei than from G0 nuclei suggested that p27, the CRM1-Ran-GTP export machinery, or both may undergo periodic post-translational changes to facilitate p27 export in early G1. The phosphorylation status of serine 10 (S10) critically regulates p27-CRM1 binding and export [111-113].

p27 was demonstrated to be a tumor suppressor protein in mice after its genetic deletion led to multi-organ hyperplasia, increased body size and susceptibility to carcinogen-induced tumors [114, 115]. In contrast to other tumor suppressors however, mutation or deletion of p27 is rare in human cancers. Instead, deregulated receptor tyrosine kinases are believed to activate Src/BCR-ABL and Ras/MEK/MAPK, or PI3K/AKT signaling which induce p27 loss or subcellular mislocalization, respectively [116, 117]. Interestingly, when p27 localizes in the nucleus it inhibits proliferation however when expressed in the cytosol it promotes cytoskeleton remodeling, suggesting a potential mecha-

nism for tumor promotion if the balance between its expression in the nucleus and cytosol was tipped toward increased cytosolic levels. Indeed, downregulation of p27 within the nucleus or its mislocalization to the cytosol consistently correlate with poor prognosis of several different cancers [117]. In one example in breast tumors, progressive p27 loss within cell nuclei has been observed during the histopathological progression of neoplasia from benign to in situ and invasive cancers and reduced nuclear p27 levels has been shown to be an independent prognostic indicator of disease relapse or death [118-120].

p53

A master regulator of the cell cycle and of genomic integrity, p53 is a tumor suppressor protein required for homeostasis of mammalian cells [121, 122]. When activated, p53 initiates several signaling pathways to arrest cell cycle progression, to repair DNA damage, and if necessary, to induce apoptosis. Approximately 50% of malignant tumors express mutant forms of p53 or have a genetic deletion of the p53 gene [123]. p53 activity is highly dependent on its subcellular localization to the nucleus, which is regulated primarily by the CRM1 nuclear export pathway [124, 125]. Interestingly, p53 and CRM1 are involved in a reciprocal regulatory loop. While nuclear p53 can repress CRM1 transcription [126], increased levels of CRM1 can promote p53 mislocalization and dysfunction, as occurs in some cancer cells [127].

In normal cells, p53 is detectable at low-levels, as it is tightly regulated by proteasome-mediated degradation and sequestration in the cytosol [125]. Both processes are mediated by one of two NES in p53: one located in the N-terminal region and one in the C-terminal region [128, 129]. A number of accessory proteins also use these latter regions to regulate p53 activity and its subcellular localization, including MDM2, PARP-1, and HPV-18 E6 [130, 131].

APC

The tumor suppressor adenomatous polyposis coli (APC) is a large protein with minimal sequence homology to other known proteins [132]. Truncating mutations in the Apc gene represents an early step in the progression of the majority of colorectal cancers, including inherited and sporadic cases [133-135]. While the mechanisms of these mutations in tumorigenesis are not fully understood, the best documented function of APC is to oppose a Wnt signal by targeting β-catenin for proteasome-mediated degradation in the cytoplasm.

A major component of the Wnt signaling pathway, β-catenin is a transcription cofactor that functions in the nucleus [136]. In normal cells, APC regulates the levels of β-catenin by promoting its nuclear export through binding to CRM1 [137, 138]. Once exported, APC is degraded in the cytoplasm through the proteolysis pathway. This process is mediated by a complex of proteins, including APC, β-catenin and the scaffolding protein Axin. Together, the complex promotes GSK-3β phosphorylation of β-catenin, targeting it for degradation. APC has been viewed as a "chaperone" protein for β-catenin, essential for its nuclear export; however, recent studies also suggest that β-catenin can engage in nuclear-cytoplasmic shuttling independent of APC and CRM1[139].

The APC protein has five different nuclear export signals, two N-terminal Rev-type NESs (NES1 and NES2) and three non-functional central signals that are deleted in the APC mutant proteins [137, 138, 140]. Of these, NES1 has the strongest signal and is the only sequence that is not truncated in the APC mutant forms. Truncated forms of human APC do accumulate in the nucleus following leptomycin B-treatment of colorectal cancer cell lines, suggesting that the nuclear-cytoplasmic shuttling ability of APC is not lost in some colorectal cancer cells. While the dynamic nuclear-cytoplasmic shuttling of APC has been suggested to be directly involved in mediating intracellular cell signaling through unknown means, APC tumor suppressor function is primarily recognized as a regulator of β-catenin [141].

Immunohistochemical studies using human colon tissue support a role for nuclear APC expression in human tumor suppression [135, 142]. While the mutant, truncated APC protein remained strongly nuclear in colon polyps [143], the frequency of APC expression within the cytoplasm increased with progression towards malignant tumors, with 60% of colon carcinomas showing cytoplasmic APC expression compared to only 4% of normal colon tissues [143]. This finding suggests that without the ability to local-

ize to the nucleus, APC is unable to maintain its regulatory tumor suppressor role.

Inhibiting CRM1 function

As many tumor suppressors and oncoproteins use CRM1 for their nuclear export and these proteins lose their normal function once they have exited the nucleus, leaving the cancer or pre-cancer cell vulnerable to constitutive growth factor and pro-survival signals, many efforts have been expended towards the development of compounds that inhibit CRM1 activity. Several of these drugs were recently discussed in an excellent review [79] therefore we will briefly mention two classes of these agents here.

Leptomycin B (LMB) was originally isolated as an antifungal antibiotic from a *Streptomyces* strain [144]. In mammalian cells, LMB resulted in cell cycle arrest at both G1 and G2 phases of the cell cycle. Upon removal of the drug, cell cycle analysis showed cells that had bypassed mitosis and become tetraploid [145]. The LMB resistance gene was identified to be a mutant of the CRM1 gene. Analyses of the mutant strongly suggested that the CRM1 protein was the molecular target of LMB [37].

LMB covalently binds to a single cysteine residue (Cys-528 in human) to inactivate CRM1 by a Michael-type addition [146]. Cys-528 is located in a central conserved region (CCR) and is conserved in LMB-sensitive organisms [146] (**Figure 2**). Crystal structure studies of the hCRM1 protein confirm that Cys-528 is located within a hydrophobic cleft, which explains why LMB-modified CRM1 cannot bind export cargoes that rely on this cleft [50, 51] (**Figure 3**). LMB is believed to compete with the NES for RanGTP-dependent formation of a stable CRM1-NES complex. LMB may also prevent the physical movement required for the required conformational change in CRM1by disrupting a hydrogen bond caused by selective alkylation at the cysteine residue, which is important for the CCR function.

Although LMB was a potent inhibitor of CRM1 and an effective cell death agent in multiple cancer cell types in vitro, it failed clinical trials in patients due to its toxicity. Therefore, several additional agents are currently in the process of development. One such class of agents is the Selective Inhibitors of Nuclear Export (SINE, KPT compounds) developed by Karyopharm Therapeutics, Inc. These are oral small molecule inhibitors which use a similar mechanism as LMB of irreversible binding to CRM1 through Cys-528. Thus far, these agents are well-tolerated in several small and large animal models and are scheduled to enter human clinical trials within the next year [147, 148].

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