Review Article

Potential effects of CRM1 inhibition in mantle cell lymphoma

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Abstract: Mantle cell lymphoma (MCL) is an aggressive histotype of B-cell non-Hodgkin lymphoma. The disease has no known cure, which prompts the urgent need for novel therapeutic agents. Chromosomal region maintenance 1 (CRM1) may play a role in human neoplasia and serve as a novel target of cancer treatment. This study summarizes MCL pathogenesis and determines the involvement of CRM1 in the regulation of several vital signaling pathways contributing to MCL pathogenesis, including the pathways of cell cycle progression, DNA damage response, phosphoinositide kinase-3, nuclear factor-κB activation, and chromosomal stability. A preclinical study is also presented to compare the CRM1 status in MCL cell lines and primary MCL cells with normal B cells, as well as the therapeutic efficiency of CRM1 inhibition in MCL *in vitro* and *in vivo*, which make these agents potential targets of novel MCL treatments.

Key Words: Chromosomal region maintenance 1 (CRM1); CRM1 inhibitor; mantle cell lymphoma

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Introduction

Mantle cell lymphoma (MCL) is an aggressive disease that has been recognized as a histotype of B-cell non-Hodgkin lymphoma (NHL), a heterogeneous group of human lymphoid neoplasms with significantly increased incidence in the United States over the past three decades (1,2). Conventional chemotherapy induces MCL remission in many previously untreated patients. However, within a few years after chemotheraphy treatment, these patients experience relapse that often leads to death with a relatively short median survival duration of 5 to 7 years (3,4). Therefore, the discovery of novel therapeutic agents for MCL with low toxicity and better treatment outcomes remains a challenge.

In MCL, the non-random t[11,14][q13;32] translocation leads to cyclin D1 overexpression, which is believed to be associated with oncogenesis. However, the overexpression of cyclin D1 alone is not sufficient for MCL development, which suggests that additional genetic events are necessary for oncogenesis (5), such as the chromosomal region maintenance/exportin1/Xpo1 (*CRM1*) gene. *CRM1* gene was first identified in the fission yeast *Schizosaccharomyces pombe* through genetic screening, and was determined to be involved in chromosomal structural control (6). CRM1 overexpression has been detected in several cancers (glioblastoma, ovarian, and cervical cancer) and has been associated with worse outcome (7-9).

The nucleocytoplasmic exchange of proteins (macromolecules larger than 40 kDa) is a spatially and temporally regulated process that involves several nucleocytoplasmic shuttling proteins. CRM1 is a nuclear protein export receptor belonging to the karyopherin β family of transport receptors, which transports target proteins across a guanosine triphosphate (GTP)-bound rat sarcoma (Ras)-related nuclear protein (RanGTP) gradient (10-13). CRM1 has a broad substrate range and mediates the export of leucine-rich nuclear export signal (NES)-bearing proteins through the nuclear pore complexes (NPCs) and the transfer of messenger RNAs (mRNAs) (14-18) (*Figure 1*).

Mechanistic studies have demonstrated the importance of the CRM1 nuclear export pathway to many NES-containing signaling molecules, including P53 (19), histone deacetylase 5 (20), protein kinase 1 (21), epidermal growth factor

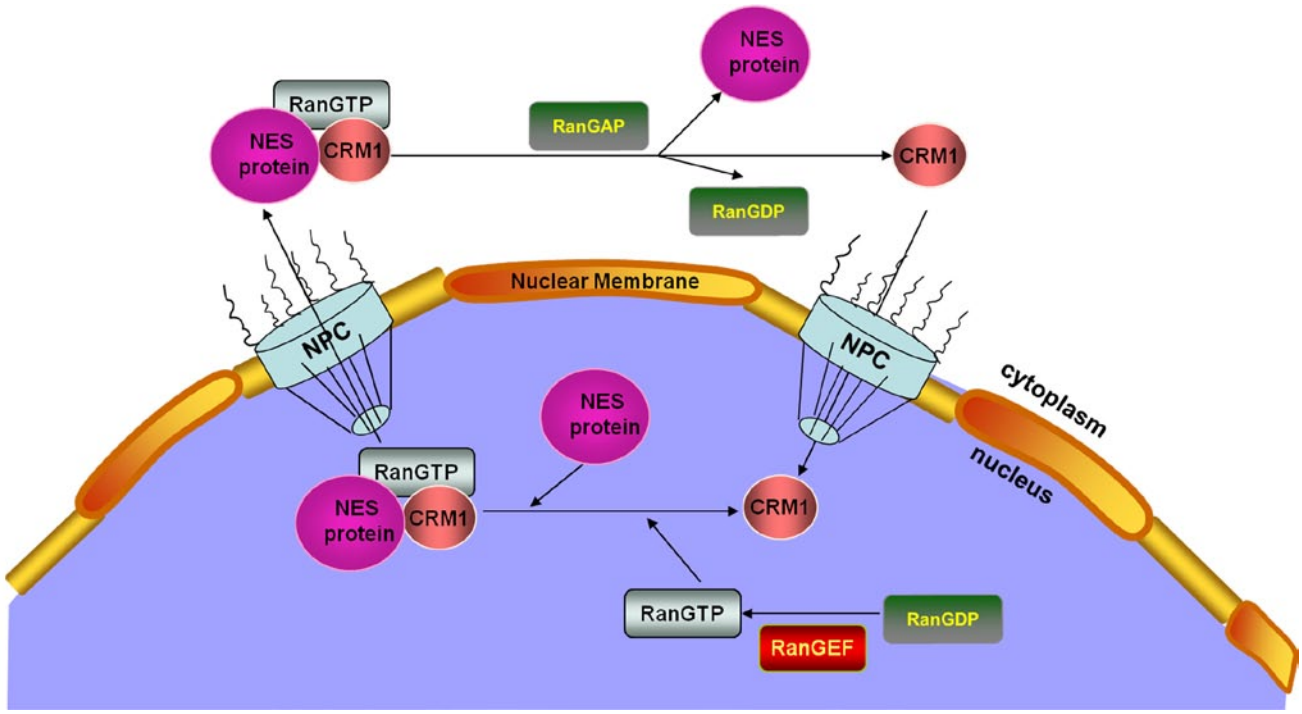

Figure 1 Nuclear export of proteins. Cargo proteins containing a nuclear export signal (NES) bind to chromosome maintenance protein 1 (CRM1) and rat sarcoma (Ras)-related nuclear protein (RanGTP) before they are exported from the nucleus through the nuclear pore complex (NPC). In the cytoplasm, the hydrolysis of guanosine triphosphate (GTP)-bound Ran (RanGTP) to guanosine diphosphate (GDP)-bound Ran (RanGDP) by a Ran GTPase activating protein promotes complex dissociation. In the nucleus, the phosphorylation of RanGDP to RanGTP by a guanine nucleotide exchange factor for Ran (Ran-GEF) allows it to reassociate with a NES-containing protein and CRM1 to restart the nuclear export process

receptor (22), and others (23,24). Given the key roles of these exported molecules in the proliferation and survival of cancer cells, including MCL cells, CRM1 could represent a new therapeutic target in MCL treatment (25-28). In this study, we summarize MCL pathogenesis and CRM1 involvement in the regulation of several vital signaling pathways contributing to MCL pathogenesis. A preclinical study is also presented to compare the CRM1 status in MCL cell lines and primary MCL cells with normal B cells, as well as the therapeutic efficiency of CRM1 inhibition in MCL *in vitro* and *in vivo*.

MCL pathogenesis

A brief overview of the relevant pathways and pathogenic mechanisms in MCL is shown in *Figure 2*. Cyclin D1 overexpression is the diagnostic hallmark in the majority of MCL patients (29). The aberrant B-cell receptor (BCR) (30) and B-cell activating factor signaling (31) both activate MCL cells. Furthermore, phosphoinositide kinase-3 (PI3K), Wnt, and transforming growth factor-β (TGF-β) signaling are also altered in MCL cells (32). Mutations in tumor suppressors such as P53 and ataxia telangiectasia-mutated (ATM) attenuate the DNA damage response in MCL cells (33). Disordered protein homeostasis and pro-apoptotic and anti-apoptotic protein imbalances also occur in MCL. Epigenomic changes in DNA methylation and histone modifications can cause genomic instability, resulting in the aberrant expression of oncogenes and/or tumor suppressor genes, thereby contributing to MCL pathogenesis (34,35).

Regulatory roles of CRM1

Subcellular localization and function of cyclin D1

Although cyclin D1 is responsible for MCL pathogenesis, cyclin D1 overexpression does not lead to the transformation of normal lymphocytes into lymphoid malignancy in nude mice (5), whereas cyclin D1 overexpression in the nucleus induces mature B-cell lymphoma in transgenic mice (36) and

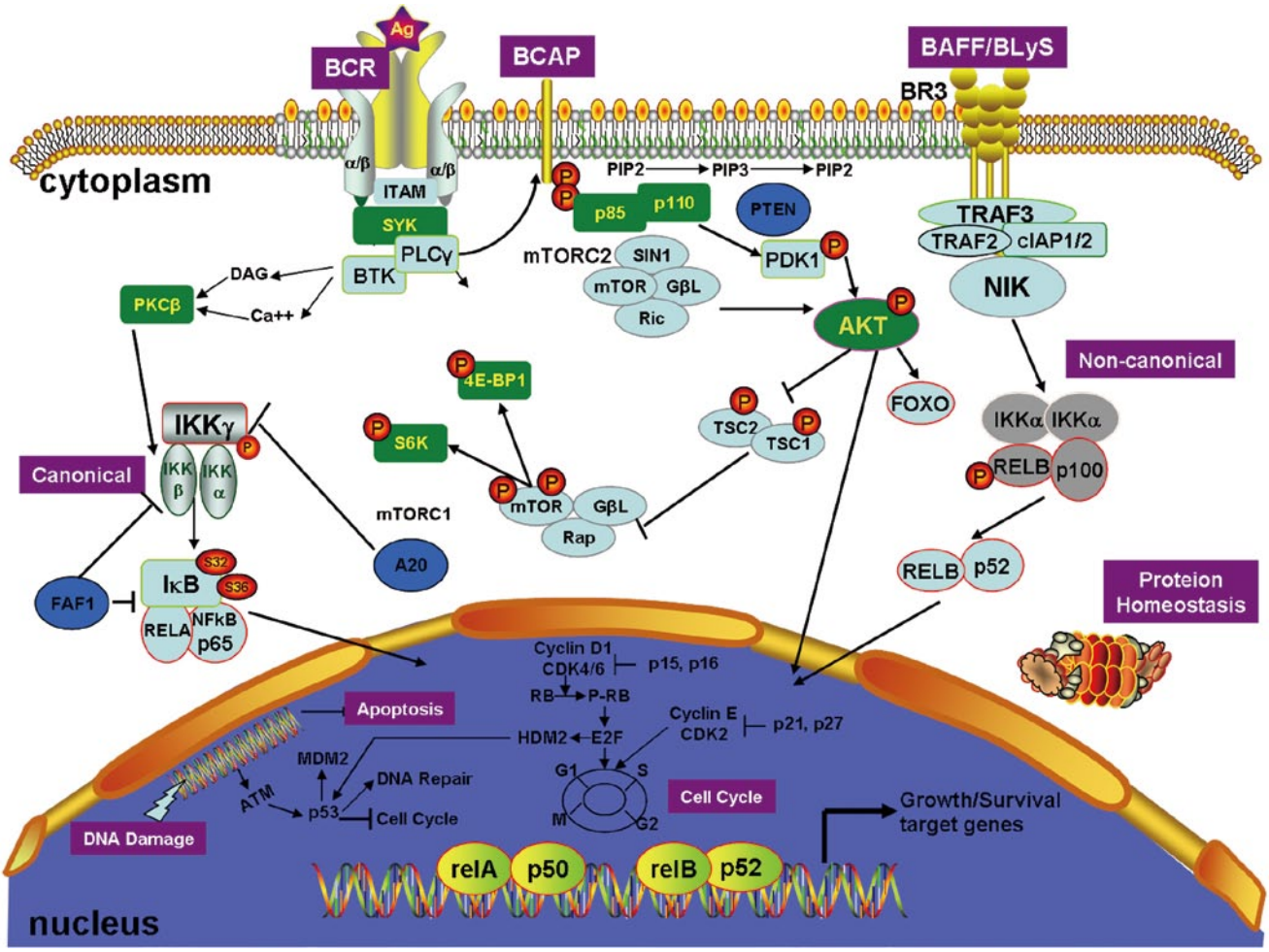

Figure 2 Major pathogenic lesions in MCL. The B-cell receptor (BCR) signaling pathway is initiated through the phosphorylation of coreceptors Igα (CD79α) and Igβ (CD79β), which recruits spleen tyrosine kinase (SYK). In turn, SYK phosphorylates several downstream kinases, including Bruton's tyrosine kinase (BTK) and phosphoinositide kinase-3 (PI3K). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) on the plasma membrane to generate the second messenger, phosphatidylinositol-3,4,5-trisphosphate (PIP3). PI3K phosphorylates phosphoinositide-dependent kinase 1 (PDK1) and the serine/threonine kinase AKT (Thr308), which activates the mammalian target of rapamycin (mTOR) and nuclear factor-κB (NF-κB) by inactivating the TSC1/2 inhibitor and by activating the inhibitor of nuclear factor-κB kinase (IKK), respectively. B-cell activating factor belonging to the TNF family (BAFF) receptor signaling cross-talks with BCR and activates NF-κB. NF-κB transcription factors form heterodimers and homodimers to activate the transcription of genes involved in cell survival, proliferation, and apoptosis. Several steps in these signaling pathways are altered in MCL. Blue symbols indicate inactivated or downregulated molecules in MCL; green symbols indicate activated or overexpressed molecules in MCL. Arrows indicate activating connections, and lines indicate inhibitory effects

drives the oncogenic transformation of murine fibroblasts in the absence of a collaborating oncogene (37,38), suggesting that the nuclear export deregulation of cyclin D1 increases its oncogenic capacity. Cyclin D1 is sequestered in the cytoplasm of mammalian cancer cells (39), where the enforced nuclear localization of cyclin D1 induces apoptosis. Thus, the subcellular localization of cyclin D1 may play a role in cell survival.

The competing processes of nuclear import and export induce cyclin D1 localization (40,41). Although the mechanisms of cyclin D1 nuclear import remain poorly characterized, the export of cyclin D1 complexes from

nucleus into cytoplasm is known to be CRM1-dependent (42). Leptomycin B (LMB), a small-molecule inhibitor of CRM1, inhibits cell cycle progression and reduces cyclin D1 expression in fission yeast and mammalian cells (43). Hence, CRM1 inhibition has therapeutic potential in patients exhibiting cyclin D1 overexpression in MCL cells.

Cell cycle progression

Cell cycle dysregulation is central to MCL pathogenesis. Cyclin D1 overexpression and abnormalities in cell-cycle inhibitory genes $p21^{WAF1}$, $p16^{INK4a}$, and $p27^{KIP1}$ have been reported in MCL (44,45). The role of CRM1 in controlling the localization and function of $P21^{WAF1}$ and $P27^{KIP1}$ could be exploited to treat MCL.

$P21^{WAF1}$ is a cyclin-dependent kinase (CDK) inhibitor that prevents cell cycle progression at the G1 phase. The nuclear localization of the unmodified $P21^{WAF1}$ is essential to elicit its anticancer functions. Two NESs are necessary to facilitate the export of $P21^{WAF1}$ from the nucleus (46,47). However, the site-directed mutation of the two NESs or by LMB blocks the $P21^{WAF1}$ nuclear export, which suggests the process is CRM1-mediated. $P21^{WAF1}$ mainly localizes to the cytoplasm in many tumor cells (48), and cytoplasmic $P21^{WAF1}$ is anti-apoptotic (49). Van der Watt *et al*. (9) found that CRM1 inhibition in cancer cells significantly reduces cell proliferation and increases apoptosis and P21 nuclear localization, which suggests that CRM1 is both a biomarker and a potential therapeutic target in MCL treatment.

$P27^{KIP1}$, a potent cell cycle inhibitor, is significantly expressed during the G0–G1 phase transition. Sanchez-Beato *et al*. found that $P27^{KIP1}$ expression correlated with the proliferative index in five MCL patients (50). Furthermore, low $P27^{KIP1}$ expression is associated with blastoid MCL, which suggests that $P27^{KIP1}$ negatively regulates the cell cycle under MCL conditions (51). The nuclear localization of $P27^{KIP1}$ enables this regulatory function. However, the nuclear export of $P27^{KIP1}$ is mediated by the CRM1 export receptor. Hence, CRM1 inhibition may restore the negative regulatory function of $P27^{KIP1}$ in MCL cell cycle progression (52).

DNA damage-response pathways

Exogenous and endogenous stress can activate ATM, a DNA damage sensor that activates the tumor suppressor P53, which, in turn, inhibits cell cycle progression and activates DNA repair mechanisms (53). P53 is often inactivated in MCL due to its deletion or mutation (33). However, P53 activity can be regulated by its subcellular localization. P53 mislocalization arising from an aberrant import mechanism, hyperactive export, or sequestration with a cytoplasmic factor, such as the glucocorticoid receptor, has been observed in several cancers, including MCL (54,55).

One promising method of controlling cell proliferation is the relocalization of P53 to the nucleus, where it becomes active. Normally, the nuclear-cytoplasmic transportation of P53 is tightly regulated. P53 contains three nuclear localization signals (NLSs), one NES in the carboxyl terminus, and one NES in the transactivation domain (19,56-58). LMB was previously used to sequester P53 in the nucleus, leading to P53 activation and cell cycle arrest and apoptosis (59). This finding signifies the importance of modulating P53 localization and provides an impetus for developing compounds that specifically target the export of P53 from the nucleus.

PI3K pathway

The constitutive activation of the PI3K pathway is key to MCL cell survival (29). In normal cells, phosphatase and tensin homolog (PTEN) (60), the cellular PI3K antagonist, can inhibit PI3K activation, resulting in the nuclear localization of forkhead box O (FOXO) transcription factors. In the nucleus, FOXO can activate the transcription of genes that promote cell cycle arrest and apoptosis (61). Thus, localizing FOXO to the nucleus is beneficial to controlling cell survival.

MCL cells frequently express the inactive phosphorylated form of PTEN that contributes to constitutive PI3K signaling. The constitutive activation of PI3K can also constitutively activate protein kinase B (AKT) (62). The constitutively activated AKT phosphorylates the FOXO transcription factors at multiple sites, thereby preventing FOXO-DNA binding and transcriptional activities, as well as promoting the CRM1-dependent export of FOXO from the nucleus (*Figure 3*) (63,64). FOXO re-localization to the nucleus, where it becomes active, is a promising method of controlling cell proliferation. Thus, CRM1 inhibition is a potential treatment for MCL.

Nuclear factor-κB activity

The transcriptional activator nuclear factor-κB (NF-κB) has been implicated in tumorigenesis and resistance therapy and

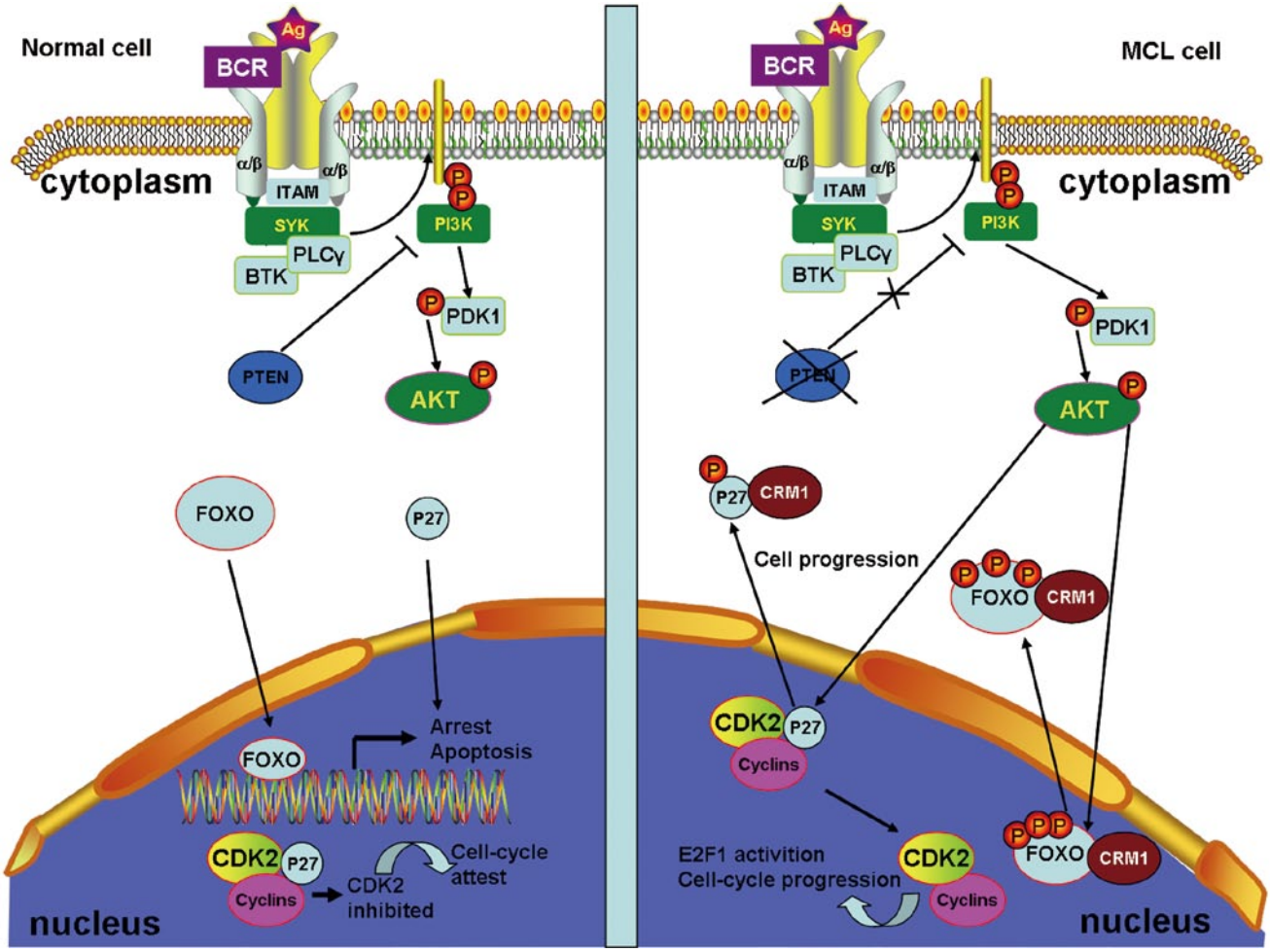

Figure 3 CRM1 regulates the PI3K signaling pathway in MCL. In normal cells, phosphatase and tension homolog (PTEN) can inhibit PI3K activation, resulting in the nuclear localization of FOXO transcription factors and P27. In the nucleus, FOXO can activate the transcription of genes that promote cell cycle arrest and apoptosis, and P27 can form complexes with cyclin-dependent kinase (CDK2) and cyclins to inhibit E2F-mediated transcription, leading to cell cycle arrest. In MCL cells without PTEN activity, PI3K/AKT signaling is active, resulting in the AKT-mediated phosphorylation of FOXO and P27. The phosphorylation of FOXO promotes its nuclear export in a CRM1-dependent manner, thereby preventing the activation of its target gene transcription. AKT also phosphorylates P27, which disrupts the nuclear localization signal (NLS) of P27 and forces it to remain in the cytoplasm. Thus, CDK2 is no longer inhibited and is free to activate E2F1 transcription factors, leading to cell proliferation

has various roles in inflammation and immune response. The molecular mechanisms of NF-κB activation in the nucleus and the roles of NF-κB in cell proliferation and apoptosis inhibition are adequately described (65-67). In normal cells, NF-κB forms a complex with the inhibitor of κB (IκB), which masks the NLS on NF-κB and prevents NF-κB translocation to the nucleus. When IκB is phosphorylated by the IκB kinase complex and degraded by the 26S proteasome, the NLS is unmasked, and NF-κB is imported into the nucleus (60,64,68). Furthermore, the p300 acetylation of NF-κB prevents NF-κB-IκB assembly and subsequent export from the nucleus, whereas the deacetylation of NF-κB enhances its interaction with IκB and promotes subsequent export from the nucleus (69).

MCL cells express constitutively activated NF-κB. The constitutive activation of NF-κB may serve as a surrogate marker for MCL, which will be valuable in assessing the effectiveness of therapeutic agents (70).

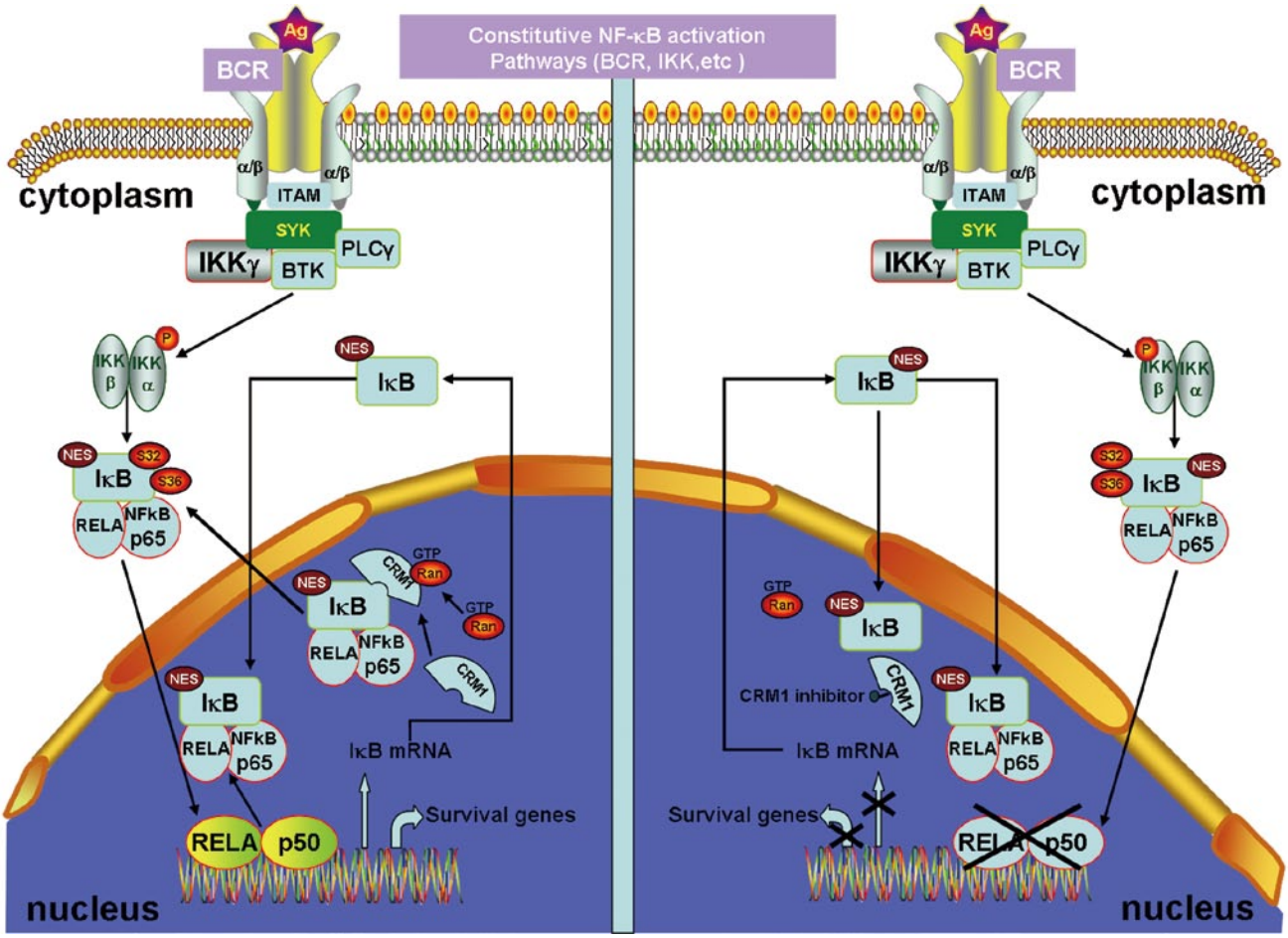

Figure 4 Inhibition of CRM1 blocks constitutive NF-κB activity and induces cancer cell death in MCL. Constitutively activated NF-κB is generally associated with a dynamic process involving the continual activation of NF-κB via an upstream activation mechanism (e.g., activation of BCR, inhibition of activated IKK, continual NF-κB-dependent synthesis of inhibitor of nuclear factor κBα (IκBα), formation of inactive NF-κB-IκBα complexes in the nucleus, and export of these inactive complexes to the cytoplasm via CRM1 and its cofactor Ran-GTP). A CRM1 inhibitor covalently modifies CRM1 to inactivate it, thereby preventing its export of NF-κB-IκBα complexes from the nucleus. This leads to the nuclear accumulation of inactive NF-κB-IκBα complexes, as well as free IκBα, with a concomitant inhibition of NF-κB function regardless of the upstream activation mechanisms involved. These events lead to the inhibition of NF-κB-dependent survival gene expression, thereby contributing to the rapid onset of apoptosis

One integral component of normal NF-κB regulation is the autoregulatory negative feedback inhibition of continuous activity through the NF-κB-directed synthesis of the inhibitor of κBα (IκBα) (71). The newly synthesized IκBα enters the nucleus, removes NF-κB from the DNA, and exports NF-κB to the cytoplasm, thereby restoring the pool of inactive NF-κB-IκBα complexes (*Figure 4*) (72). A CRM1-dependent pathway mediates IκBα nuclear export (73). Therefore, the perturbation of the CRM1-dependent nuclear export of IκBα may attenuate constitutively activated NF-κB and cause immediate apoptosis in different cancer types (74). Therefore, the perturbation of the CRM1-dependend IκBα nuclear export induces apoptosis in MCL.

Centrosome duplication

One potential therapeutic target in MCL is centrosome aberration. Blastoid MCL is characterized by high mitotic rate of MCL cells and poor prognosis, regardless of its

cytomorphological subclassification (1,75-78). It contains more chromosomal imbalances than non-blastoid MCL and exhibits high-level DNA amplifications. Moreover, blastoid MCL tends to be a tetraploid (78,79), a rare phenomenon occurring in B-cell neoplasms (80).

Chromosomal aberrations, which are common features of malignant neoplasias, can be induced by impaired DNA damage response pathways, mitotic checkpoint alterations, or centrosome aberrations. As the major microtubule-organizing centers in animal cells, centrosomes play a significant role in cell cycle progression, spindle formation, and cytokinesis (81). Neben *et al*. (82) found that centrosome aberrations occur more frequently in near-tetraploid MCL than in diploid MCL, suggesting that centrosome aberrations may play a role in MCL tetraploidization and are thus potential therapeutic targets in MCL, especially blastoid MCL.

In principle, centrosome aberrations could arise *de novo* or through the over-duplication of centrosomes within a single cell cycle, through aborted cell division, or through cell fusion (83). However, the mechanisms underlying the regulation of centrosome duplication are poorly understood. Some studies suggest that the Ran/CRM1 complex regulates nucleocytoplasmic transportation and is independently involved in mitotic spindle assembly. CRM1 may regulate the fidelity of centrosome duplication (84) by acting as a licensing factor to prevent unscheduled duplication. Hence, CRM1 inactivation either by a CRM1-specific inhibitor, such as LMB, or through the interaction of the hepatitis B virus HBx protein with its NES motif results in supernumerary centrosomes (84,85), which suggests that CRM1 may negatively regulate the initiation of centrosome duplication, possibly through its association with NES-containing proteins (*Figure 5*) (86,87).

Spindle assembly

During mitosis, the supernumerary centrosomes can form multipolar spindles, which occur in many tumor types and are believed to contribute to chromosomal instability and tumorigenesis (83,88). However, some studies have shown that multipolar divisions and the resulting chromosomal instability undermine cell viability, frequently leading to cell death (89-92). Many cancer cells induce supernumerary centrosome clustering into two spindle poles, thereby enabling bipolar division, to avoid cell death. As the phenotype is specific to cancer cells, inhibiting centrosome clustering may target cancer cells selectively without affecting healthy cells.

The centrosome is composed of centrioles and pericentriolar material. A key component of the pericentriolar material and centrioles is pericentrin (PCNT2), a large conserved coiled-coil protein (93-95) that anchors γ-tubulin to the centrosomes and plays a key role in microtubule nucleation and mitotic spindle organization (93,96,97). Neben, *et al*. found that the expressions of four centrosome-associated proteins (PCNT2, calcium/calmodulin-dependent protein kinase II, and γ-tubulin complex-associated proteins 3 and 4) were high in MCL (98), suggesting that MCL harbors aberrant spindle assembly.

CRM1 located at centrosomes by its N-terminal CRM1, and importin beta etc. domain can interact with and regulate the localization and function of PCNT2 (99). Given the ability of PCNT2 to serve as a multifunctional scaffold for anchoring a wide range of centrosome proteins (100), it is involved in essentially all centrosome functions that center primarily around cell cycle regulation and microtubule organization. Increased PCNT2 levels can alter centrosome number, clustering, and function, thereby altering mitotic spindle organization and function, as well as causing chromosome missegregation. Centrosome clustering may enable cancer cells with multiple centrosomes to undergo relatively normal cell divisions. However, this likely leads to genetic instability, a known contributor to carcinogenesis (101-103). CRM1 inactivation can cause unscheduled centriole splitting that results in multipolar spindles. Hence, CRM1 safeguards the bipolar spindle formation by preventing unscheduled centriole splitting during mitosis (104).

Leber *et al*. found that the components of the chromosomal passenger complex are necessary for centrosome clustering (105). The chromosomal passenger complex is composed of aurora B and its regulatory subunits, inner centromere protein, survivin, and borealin. Survivin and aurora B are involved in the development and progression of human tumors and are thus potential targets for novel anticancer therapies (106,107). In MCL, survivin is commonly expressed in a nuclear and mitotic pattern, and its expression levels are strongly associated with tumor proliferation and patient survival (108). Dynamic nucleocytoplasmic transport regulates the subcellular localization and function of survivin and aurora B (109). Survivin and aurora B are actively excluded from the nucleus by a CRM1-mediated mechanism (110). The spatial and functional regulation of survivin by LMB abolishes

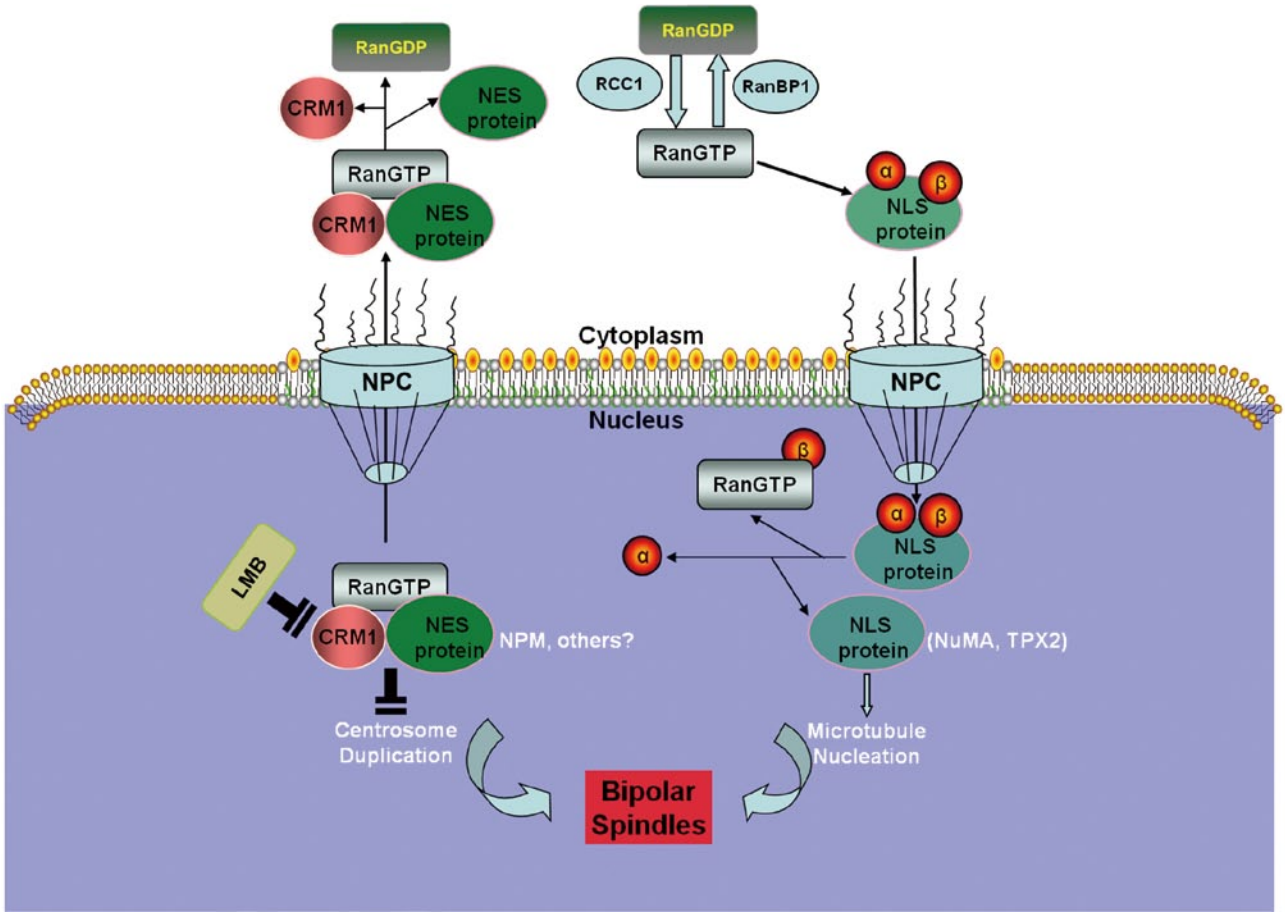

Figure 5 Ran/CRM1 network: nucleocytoplasmic transport and mitotic spindle assembly. The small GTPase, Ran, shuttles between an inactive GDP state and an active GTP-bound state by interacting with Ran-binding protein 1 (RanBP1) and regulator of chromosome condensation (RCC1), respectively. In its GTP-bound state, Ran can interact with importin receptors α and β to promote the transport of proteins containing nuclear localization signals (NLS) from the cytoplasm to the nucleus. The transport of certain NLS-containing proteins, such as the nuclear mitotic apparatus protein (NuMA) and target protein for XKIP2 (TPX2), can promote microtubule nucleation. Ran-GTP can also interact with CRM1, which binds to proteins containing NES. LMB interacts with and inactivates CRM1 through its NES, leading to centrosome overduplication and multipolar spindles. Other NES-containing substrates that bind to CRM1, such as nucleophosmin (NPM), may have tumor-suppressing effects and function as licensing factors to regulate centrosome duplication during the cell cycle

the cytoprotective effect of survivin towards apoptotic executors, resulting in cell apoptosis (*Figure 6*) (111).

Novel CRM1 inhibitors for MCL therapy

In a previous study (112), we identified several novel CRM1 inhibitors that show potential for MCL treatments. We also found that CRM1 expression is higher in MCL cell lines and primary MCL cells than in normal B lymphocytes. Inhibiting CRM1 with small interfering RNA consequently inhibited MCL cell growth. These findings suggest that CRM1 may play a key role in the pathophysiology of MCL cells. In addition, targeting CRM1 in MCL may have therapeutic value.

Other researchers found that LMB binds covalently to cysteine 528 (Cys528) in CRM1 through a Michael-type addition reaction and abrogates the interaction between CRM1 and its cargo protein (13,113,114). Although LMB possesses strong antitumor activity *in vitro*, phase I trials of LMB were discontinued because of its toxicity and lack

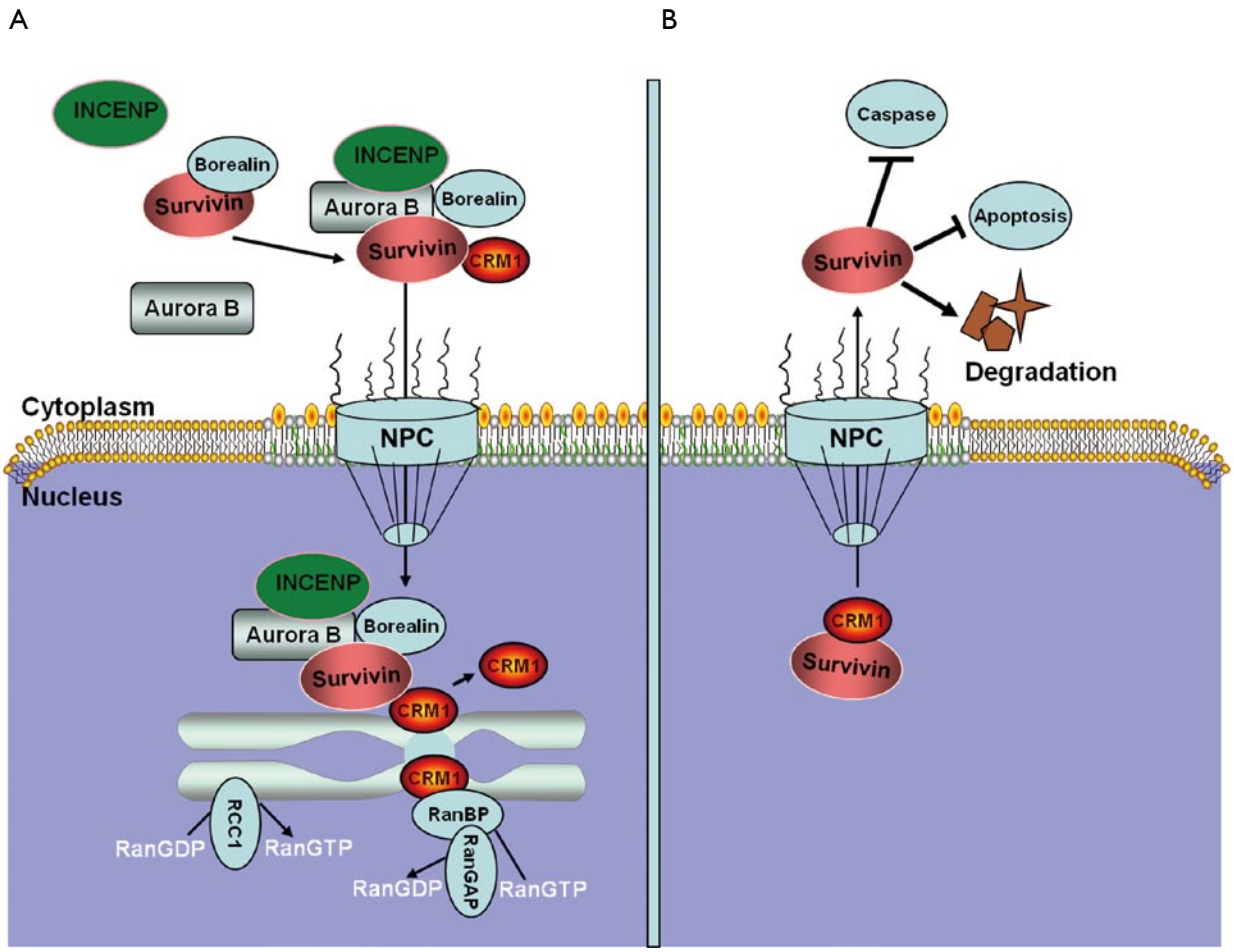

Figure 6 CRM1/survivin axis supports the dual activity of survivin. A. At the beginning of mitosis, the CRM1-survivin interaction is critically involved in the tethering of the chromosomal passenger complex (CPC) to the centromere; B. At the end of mitosis, upon reassembly of the nuclear envelope, CRM1 mediates the removal of survivin from the nucleus, which may facilitate proteasome degradation in the cytoplasm. In interphase cells, nuclear export promotes a high cytoplasmic and mitochondrial concentration of survivin to counteract pro-apoptotic stimuli

of apparent efficacy within the tolerable dose range (115). Mutka *et al*. (116) noted that LMB has off-target effects against proteins other than CRM1 that might contribute to its toxicity. The finding that the inhibition of CRM1 itself was not the cause of LMB toxicity is promising in terms of the development of CRM1-targeted anticancer drugs.

In order to efficiently discover novel small-molecule selective inhibitors of nuclear export (SINEs; also known as KPT-SINE compounds) that block CRM1-dependent nuclear export, Karyopharm Therapeutics applied a virtual screening workflow based on a combination of protein modeling and simulations, physicochemical filters, and high-throughput molecular docking (117,118). These CRM1-specific inhibitors are similar to the N-azolylacrylate structures developed by Daelemans *et al*. (119). KPT-SINE compounds are water-soluble, irreversible inhibitors of CRM1 that bind to the reactive site of the Cys528 residue. Azmi *et al*. (120) demonstrated that KPT-SINE compounds can induce apoptosis in resistant NHL cell lines and corresponding xenograft models. Their study verifies CRM1 as a potential therapeutic target in NHL irrespective of the functional status of P53. We found that using KPT-SINE compounds to inhibit CRM1 in MCL cell lines and primary MCL cells could significantly inhibit MCL cell growth and induce apoptosis. The oral administration of KPT-276, a KPT-SINE compound, significantly suppressed

tumor growth in an MCL-bearing severe combined immunodeficient mouse model without severe toxicity (112). These findings suggest that CRM1 inhibitors are a potential novel therapy for MCL patients.

Conclusions

In conclusion, CRM1 can regulate MCL cell proliferation, cell cycle progression, DNA damage response, and chromosomal stability, making it a potential therapeutic target in MCL treatment. We found that CRM1 is overexpressed in MCL cells and that CRM1 inhibition using small interfering RNA or CRM1 inhibitors, such as KPT-SINE compounds, significantly inhibits MCL cell growth both *in vitro* and *in vivo*, making these agents potential novel treatments for MCL. Clinical studies to evaluate the therapeutic effects of KPT-SINE compounds in MCL patients are warranted.

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