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Nuclear transport as a target for cell growth

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The function of many key proteins and transcription factors involved in cell growth can be regulated by their cellular localization. Such proteins include the tumor suppressor p53 and the nuclear factor κB. Although the idea of trapping such proteins in either the nucleus or cytoplasm has been introduced as a potential therapeutic target, only two nuclear transport inhibitors have been reported. Here, we explore the roles of small-molecule inhibitors that cause target proteins to sequester in either the nucleus or cytoplasm. Methods of artificially targeting proteins to the nucleus or cytoplasm using peptide aptamer technology are also discussed.

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▼ Screens for novel anticancer therapies at the cellular level have traditionally relied on testing compounds for their ability to induce apoptosis or block cell proliferation in a spectrum of cancer cell-lines. Although these types of screens are powerful and effective, they do not always identify the molecular targets of such compounds, nor do they reveal any new biology that might be beneficial for future drug development. Recent advances in the study of cancer cell biology have indicated an increasing number of oncogenes and tumor suppressors as important intervention points for next-generation anticancer drugs. A crucial aspect of gene function is the subcellular site-of-action of the protein. For example, transcription factors are downregulated when localized to the cytoplasm, but activated when translocated to the nucleus.

Recently, advances in high-content imaging have expanded the scope of HTS. It is now possible to screen for cellular phenotypes such as nuclear- versus cytoplasmic-localization of a target protein in several thousand samples. This review examines the possibilities for modulating protein localization as a strategy for the discovery of novel anticancer agents.

Nuclear transport of proteins

One of the hallmarks of eukaryotic cells is the containment of DNA in the cell nucleus, where transcription and replication are separated from protein synthesis in the cytoplasm. Transport of molecules between the cytoplasm and nucleus occurs across the nuclear envelope through the nuclear pore complex (NPC), a large protein structure of ~125 megadaltons (MDa) that enables the passage of a variety of complexes, up to 120 kDa, the size of the large ribosomal subunit [1]. In principle, molecules of 60 kDa can diffuse freely across the NPC; however, in reality, the trafficking of several such molecules is tightly controlled [2].

In general, proteins to be imported or exported from the nucleus contain either a nuclear localization signal (NLS) or nuclear export signal (NES), which is recognized by a receptor and carried through the NPC [3,4]. Once in the correct compartment, the protein cargo is released and the receptor recycled for another round of transport. Ran is the small Ras-like GTPase crucial for maintaining the direction of transport. The asymmetrical distribution of the Ran guanine nucleotide exchange factor (RanGEF) to the nucleus, and the Ran GTPase-activating protein (RanGAP) to the cytoplasm, establishes a gradient of the nucleotide-bound state of Ran. This results in a high RanGTP concentration in the nucleus; the maintenance of this gradient is essential for nuclear transport and cell viability.

Members of a growing family of transport receptors carry proteins into and out of the nucleus and bind preferentially to Ran in its GTP-bound form. For protein export, the exportin CRM1 forms a complex with the NES-containing cargo and RanGTP in the nucleus (Fig. 1). Once the complex is translocated into the cytoplasm, the RanGAP promotes

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hydrolysis of RanGTP to RanGDP, causing the NES-containing cargo to be released. For the nuclear import of many NLS-containing proteins, the adaptor protein, importin α, recognizes and binds the NLS-containing cargo (Fig. 1). The import receptor, importin β, can then bind to importin α, and this complex translocates across the nuclear envelope through the NPC. Once in the nucleus, RanGTP preferentially binds to importin β, causing the release of the NLS-containing cargo.

Many proteins for import are not recognized by the importin-α or -β receptor, but are recognized by other receptors related to importin β. There are at least 21 potential importin β family members in humans [5]. The full spectrum of cargos for each import receptor is not known, but some might show sufficient specificity to be considered as targets for regulation.

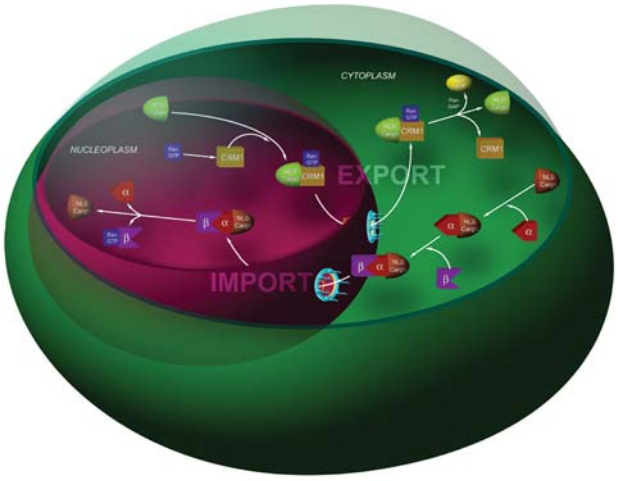

Figure 1. Protein nuclear export involves complex formation of CRM1 with the nuclear export signal (NES)-containing cargo and RanGTP in the nucleus. After translocation through the nuclear pore complex (NPC), RanGAP promotes hydrolysis of RanGTP to RanGDP, causing the release of the NES-containing cargo. For nuclear import, the nuclear localization signal (NLS)-containing cargo forms a complex with importin α and importin β before translocation into the nucleus. Preferential binding of importin b with RanGTP causes the release of the NLS-containing cargo.

Nuclear export inhibitor leptomycin B as a cancer therapeutic

Isolated from a *Streptomyces* strain, leptomycin B (LMB) has, until recently, been the only known small-molecule inhibitor of nuclear transport. Leptomycin B is an unsaturated branched-chain fatty acid (Fig. 2) and has been shown to have antifungal, antibacterial and anti-tumor activity [6–8]. Furthermore, LMB causes cell-cycle arrest at stages G1 and G2 of the cell cycle in yeast and mammalian cells [9]. Leptomycin B was first implicated as an inhibitor of nuclear export in a screen for compounds that block the export of HIV Rev [10]. As Rev contains an NES, it was possible that LMB blocked not only NES-mediated Rev export, but also general cellular protein export.

Work performed primarily in yeast over the past two decades has identified CRM1 as the target of LMB. CRM1 was first identified from a genetic screen for altered higher chromosome structure in cold-sensitive mutants of *Schizosaccharomyces pombe* [11]. Although the function of CRM1 was not known, it was linked to LMB when Nishi *et al.* cloned a mutant form of the CRM1 gene that conferred LMB resistance in yeast [12]. CRM1 was subsequently identified as an NES and nuclear export receptor, and LMB as an inhibitor of CRM1–NES binding [13–16]. Consequently, this discovery helped identify CRM1 as the export receptor for all NES-mediated nuclear transport. Leptomycin B has been shown to bind covalently to CRM1 [17], and *S. pombe* mutants no longer hypersensitive to LMB contain a single amino-acid substitution in CRM1, where a serine residue replaces cysteine-529 [18]. This single cysteine residue provides LMB sensitivity in CRM1 because the α,β-unsaturated δ-lactone in LMB can undergo a Michael-type reaction by the sulfhydryl group on cysteine-529 [18] (Fig. 2). Once bound to LMB, CRM1 cannot complex with the NES of export cargos.

Daelemans *et al.* have recently identified a synthetic compound that disrupts CRM1–NES binding in a screen for inhibitors of HIV Rev function. PKF050–68 contains an α,β-unsaturated ketone and, like LMB, probably targets CRM1 at the reactive cysteine residue [19].

Since its discovery, LMB has become an important 'tool compound' for studying the regulation of nucleocytoplasmic shuttling proteins. Leptomycin B is highly specific to CRM1, and highly potent, as exemplified by its ability to block CRM1–NES binding at tens-of-nanomolar *in vitro* [14,15]. Even before CRM1 was identified as the target of LMB, the anti-tumor activity of LMB was explored and led to its testing as a potential cancer therapeutic in a phase I clinical trial. The trial revealed LMB as having a high toxicity profile; following intravenous administration of the drug, patients suffered from nausea, vomiting, anorexia and malaise, regardless of the administration schedule [20]. Thus, no further clinical development was recommended and has not taken place.

Despite negative results from the phase I clinical trial, increased understanding of the molecular mechanisms-of-action of LMB and similarly acting compounds has meant that their use as anticancer agents has been re-visited more than once [21]. Trapping effectors, such as transcription factors, protein kinases or mRNA, has been suggested as a

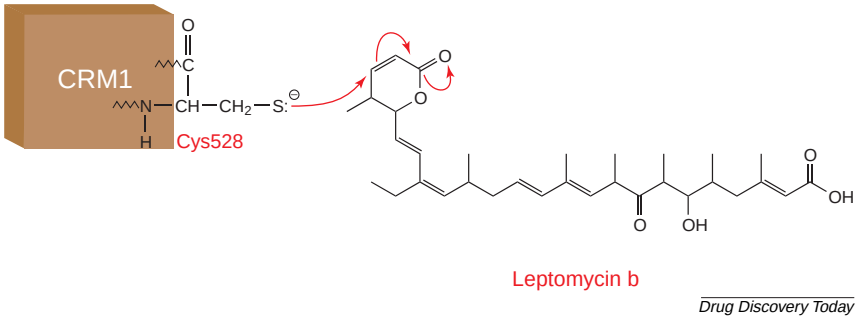

Figure 2. Leptomycin B (LMB) is an unsaturated branched-chain fatty acid and can undergo a Michael-type addition by Cysteine-529 on the exportin CRM1. This covalent modification inhibits binding between CRM1 and the nuclear export signal (NES)-containing cargo.

way of treating aberrantly growing cells. For example, an active BCR–ABL trapped in the nucleus by LMB can result in cellular apoptosis [22]. BCR–ABL is a tyrosine kinase fusion protein expressed in chronic myelogenous leukemia (CML) cells. Fusion of the cellular breakpoint cluster region gene (Bcr) with the Abelson murine leukemia oncogene (Abl) results in a constitutively active non-receptor tyrosine kinase that stimulates several signal transduction pathways. Currently, CML is treated with the drug STI571 (Gleevec), an inhibitor of BCR–ABL. Treatment of cells with STI571 and LMB results in the nuclear entrapment of BCR–ABL. Furthermore, upon restoration of BCR–ABL function by washing-out STI571, treated cells undergo cellular apoptosis [22]. Although LMB initially failed in clinical trials, its use in conjunction with other therapeutics could prove to be a potent and novel method of eliminating cancer cells.

Targeting nuclear localization of key transcription factors in disease

Several well-characterized transcription factors have been implicated in tumorigenesis. As transcription occurs in the cell nucleus, the activity of these factors can be regulated by their subcellular localization [23,24]. Thus, identifying small molecules that affect the localization of transcription factors can be an effective target for the control of unwanted cell growth.

NFκB

Nuclear factor κB (NFκB) is a transcriptional activator involved in inflammatory and immune responses in the cell. It has been implicated in tumor development and anticancer drug resistance, and anticancer drugs targeting NFκB have become of growing interest [25,26]. Nuclear factor κB can activate several different classes of target genes involved in negative feedback, immunity, cell proliferation and apoptosis. In addition, it is constitutively activated in cancer cells, aiding tumor resistance to radiation and anticancer drug treatments [25]. It can promote cell proliferation by activating the expression of cyclin D1 [27,28]. In addition, it transcriptionally activates genes that block TNF-α-induced cell death [29–31]. Thus, preventing NFκB activation inhibits cell-cycle progression and promotes apoptosis.

In unstimulated cells, NFκB is found in the cytoplasm where it is inactive and complexed with the inhibitor of nuclear factor κB (IκB). Binding with IκB masks the NLS on NFκB and prevents its nuclear import [32–34]. When factors such as TNF-α, CD-40 ligand, or interleukin-1 stimulate cells, IκB is phosphorylated by the Iκκ complex and then degraded by the 26S proteosome. No longer masked by IκB, the NLS on NFκB is available to form a complex with the import machinery, and NFκB translocates into the nucleus where it activates its target genes (Fig. 3).

There are several points within the NFκB signaling pathway that could block NFκB activation. These include: (1) the point where stimuli initiate the cascade; (2) the level of IκB phosphorylation and degradation; (3) inhibition of NFκB nuclear translocation; and (4) counteracting the nuclear activity of NFκB (Fig. 3). Many of the NFκB inhibitors identified to-date have the effect of blocking its nuclear translocation. Proteasome inhibitors, such as lactacystin, stabilize IκB, thereby maintaining IκB-bound NFκB in the cytoplasm [25]. Similarly, inhibitors of IκB phosphorylation, such as nitric oxide and aspirin, also prevent nuclear import of NFκB by maintaining a high concentration of IκB in the cytoplasm [25]. Known inhibitors that specifically block NFκB nuclear translocation include dehydroxymethylepoxyquinomicin (DHMEQ) and o,o′-bismyristoyl thiamine disulfide (BMT), although the mechanism of how these compounds block NFκB nuclear translocation is still unknown [35,36]. In Jurkat cells, DHMEQ has been shown to block NFκB induction by TNF-α and increase apoptosis [36]. In addition, DHMEQ blocks the activation of NFκB in T-cell leukemia cells [37]. Furthermore, BMT inhibits the nuclear transport of NFκB as well as the HIV-1 transactivator, Tat [35]. Thus, inhibiting nuclear import of NFκB has been a potent method of preventing NFκB activation, whether the molecular target is involved in phosphorylation, IκB

degradation, or active nuclear transport of NFκB. As NFκB nuclear transport occurs downstream in the signaling pathway, targeting its nuclear translocation can also reveal upstream mechanisms involved in NFκB activation.

p53

The activity of the tumor suppressor p53 can be regulated by its subcellular localization. However, unlike NFκB, p53 activation promotes cell-cycle arrest and apoptotic cell death. Thus, localizing p53 to the nucleus is desirable for the control of cell survival. Mislocalization of p53 has been observed in several cancer cells and tumors arising from an aberrant import mechanism, hyperactive export, or sequestration with a cytoplasmic factor such as the glucocorticoid receptor [38,39]. Re-localizing p53 to the nucleus where it is active, is a promising method of controlling cell proliferation.

Together with its subcellular localization, p53 activity and stability is controlled by MDM2, an E3 ubiquitin ligase. In addition to targeting p53 for degradation, MDM2 also regulates its own protein levels, thereby establishing an auto-regulatory feedback loop with increases in p53 activity [40]. Many recent advances have been made in the study of how MDM2 ubiquitinates p53 in relation to p53 subcellular localization and p53 nucleocytoplasmic shuttling. Both p53 and MDM2 shuttle in and out of the nucleus, and p53 nuclear localization and export signals have been identified at the C-terminus [41,42]. Recently, a new NES in the N-terminus of p53 has been identified [43].

Elucidating the mechanisms involved in p53 export can reveal different ways of maintaining p53 in the nucleus. Several models of p53 export have been proposed, including: (1) MDM2 as a chaperone, actively shuttling p53 from the nucleus to the cytoplasm [41]; (2) tetramerization of p53, thereby masking its C-terminal NES and preventing its nuclear export [42]; (3) ubiquitination by MDM2, promoting p53 export [44,45]; and (4) phosphorylation of p53 at the N-terminus by DNA-damage-induced protein kinases, inhibiting the N-terminal NES of p53 [43]. It has also been suggested that phosphorylation by DNA-damage-induced kinases not only blocks export, but also prevents MDM2-binding at the N-terminus [43]. Without MDM2 binding, p53 remains stabilized and active in the nucleus. It is possible that p53 uses one or more of these mechanisms for the regulation of its nuclear export under different conditions. Further research into understanding how p53 is exported will be important in establishing methods to maintain and activate p53 in the nucleus.

In addition to targeting p53 export, p53 import mechanisms can reveal novel ways of activating p53.

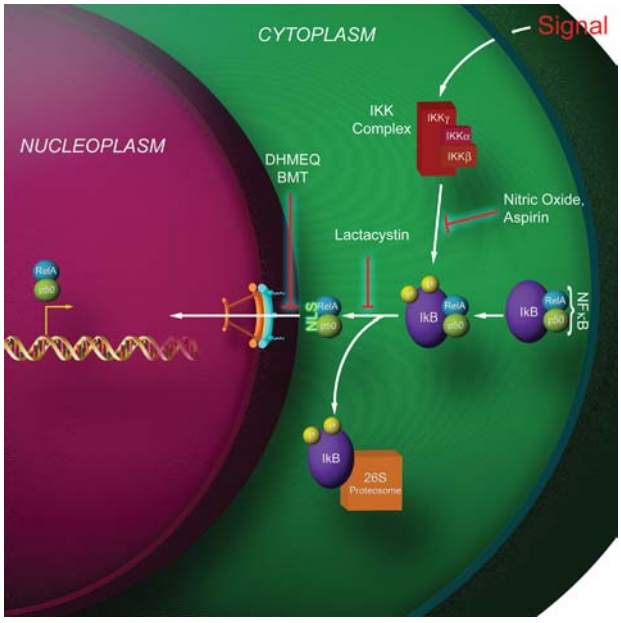

Figure 3. Phosphorylation on the inhibitor of nuclear factor κB (IκB) by the Iκκ complex results in IκB degradation and unmasking of the nuclear localization signal (NLS) on nuclear factor κB (NFκB). Nuclear factor κB can then be imported into the nucleus where it activates its target genes. Several points of inhibition within the pathway are targeted by compounds such as aspirin and lactacystin, resulting in the cytoplasmic sequestration of NFκB.

Microtubules and the dynein motor protein are involved in facilitating p53 nuclear import [46]. Furthermore, mutations in the general protein import machinery can lead to p53 mislocalization. For example, a truncated form of importin α has been found in a human breast cancer cell-line that lacks the putative NLS binding domain [38]. Thus, in these cells, truncated importin α cannot bind to the NLS of p53, which might result in the accumulation of p53 in the cytoplasm.

Leptomycin B has also been used to trap p53 in the nucleus, leading to p53 activation and resulting in cell-cycle arrest and apoptosis [47]. Treatment of human foreskin primary fibroblasts with LMB led to the induction of p53 transcriptional activity, increased p53 levels, and increased the accumulation of p53 and MDM2 in nuclear aggregates [47]. Furthermore, LMB together with actinomycin D can reactivate p53 and prevent its degradation by HPV E6 protein in cervical carcinoma cells infected with human papillomavirus [48]. Thus, results achieved with a general export blocker provide an essential proof-of-concept for the importance of modulating p53 localization, and an impetus for the development of compounds that specifically target p53 export.

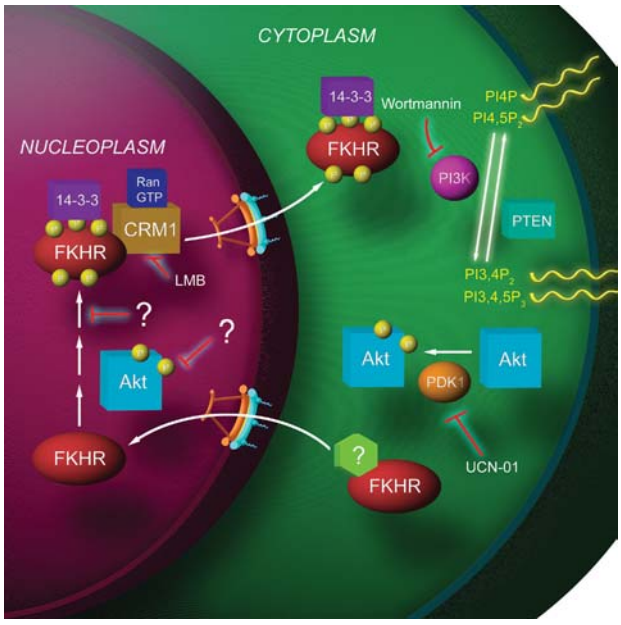

Figure 4. Phosphoinositide 3-kinase (PI3K) signaling in the context of FKHR nuclear transport: phosphorylation of PI4P and PI4,5P2 by PI3K results in the recruitment of protein kinase B (Akt) to the plasma membrane. Akt is then activated by PDK1 and can phosphorylate FKHR in the nucleus. How FKHR is imported into the nucleus is still unknown, although researchers are beginning to elucidate the mechanism of FKHR hyper-phosphorylation. A hyper-phosphorylated FKHR can form a complex with 14-3-3, CRM1, and RanGTP, and be exported into the cytoplasm. Several points of inhibition exist in the pathway to sequester FKHR to the nucleus, including kinase activity and general transport machinery. In addition to wortmannin and UCN-01, which block PI3K and PDK1 activity, respectively, researchers have identified inhibitors of Akt as well as other as-yet unknown kinases involved in the phosphorylation of FKHR.

PI3K–Akt pathway and Forkhead transcription factors

The PTEN lipid phosphatase, first identified as the phosphatase and tensin homolog deleted on chromosome ten, acts as a tumor suppressor and negative regulator of cell growth and survival driven by the phosphoinositide 3-kinase–protein kinase B (PI3K–Akt) signaling pathway. Mutations in PTEN have been implicated in tumors as well as in Cowden Disease – a hereditary disease marked by a high pre-disposition for breast and thyroid cancers [49]. In addition, PTEN mutations have been found in cancers such as glioblastoma multiforme, endometrial cancer, prostate cancer, melanoma, lymphoma, and bladder and renal cell carcinoma [49].

Inhibition of the PI3K–Akt signaling pathway can control aberrant cell growth. Formation of the PI3K products, PI3,4,P_2 and PI3,4,5,P_3, increases in growing and stimulated cells [50]. PTEN antagonizes PI3K signal transduction by de-phosphorylating the PI3K phosphorylation products at the D3 position of the inositol ring [51]. PI3,4,P2 and PI3,4,5,P3 recruit Akt, a protein serine/threonine kinase and effector of PI3K signaling, to the plasma membrane through interaction at the Pleckstrin homology (PH) domain on Akt. Phosphorylation by 3-phosphoinositide-dependent protein kinase-1 (PDK1) on Akt then results in Akt activation. A hyperactive Akt can result in the suppression of cellular apoptosis by inhibiting cell-death effectors [52]. Phosphorylation targets of Akt, such as the Bcl2-antagonist of cell death (BAD), caspase 9, and the Forkhead family of transcription factors, become inactive when phosphorylated [52] (Fig. 4).

Mammalian members of the Forkhead transcription factor family include AFX, forkhead in rhabdomyosarcoma (FKHR) and FKHRL1. These proteins are orthologs of the *Caenorhabditis elegans* Forkhead transcription factor, Daf-16. In the nematode, activation of the PI3K–Akt pathway can lead to exit from the dauer stage in larvae, as well as promoting aging in adults [53,54,55]. Activation of Akt and the subsequent detachment of Akt from the plasma membrane result in its import into the nucleus, where it is free to phosphorylate and inhibit the Forkhead transcription factors [56,57].

Forkhead activity is regulated by both phosphorylation and subcellular localization. When phosphorylated, it is localized to the cytoplasm and is thus inactive; when non-phosphorylated, it is located in the nucleus and is active [58–61]. A triple alanine mutant form of FKHR, which cannot undergo phosphorylation, localizes to the nucleus, and cells expressing this mutant arrest at G1 [62]. Thus, sequestering Forkhead in the nucleus might represent a method for treating PTEN mutant cells. Moreover, inhibition of upstream kinases in the PI3K–Akt pathway can regulate Forkhead nuclear localization. For example, treatment with the PI3K inhibitor, wortmannin, results in Forkhead nuclear retention [63]. Other potential kinase targets include Akt and PDK1. Furthermore, the kinases, SGK and DYRK1 have recently been implicated in Forkhead phosphorylation [64,65]. The molecular mechanisms regulating Forkhead nuclear transport are still not completely understood. However, in addition to phosphorylation, the 14-3-3 binding protein is involved in Forkhead nuclear export and retention in the cytoplasm [66,67].

Recently, two anticancer drugs in clinical trials, PKC412 and UCN-01, have been implicated in targeting the PI3K–Akt survival pathway [68,69]. These compounds are staurosporine derivatives and known protein kinase C (PKC) inhibitors, but also exert inhibitory activity within the PI3K pathway (and possibly other kinase pathways). UCN-01 (7-hydroxystaurosporine) inhibits PDK1 activity

at 33nM [68]. It is not known whether these compounds cause cytotoxicity through activation of Forkhead transcription factors or through other death promoters such as BAD and caspase 9. Interestingly however, several staurosporine-related compounds have been isolated in a chemical genetic screen for inhibitors of FKHR nuclear export (T.R. Kau and P.A. Silver, unpublished results). These recent discoveries show that the PI3K–PDK1–Akt survival pathway is an important target for the control of cell growth through Forkhead phosphorylation, nuclear localization, and activation.

Moving proteins with peptide aptamers

In contrast to inhibiting the endogenous signaling pathways of a cell to re-localize effectors of cell death or cell growth, peptide aptamers enable the active targeting of effectors to the nucleus or cytoplasm. Analogous to monoclonal antibodies, peptide aptamers are small proteins that contain a structurally constrained variable region of ~20 amino acids, expressed as part of an inert scaffold such as thioredoxin or green fluorescent protein [70]. Peptide aptamers that have been created include those against CDK2, E2F, and the human papillomavirus E6 oncoprotein [71–73]. These aptamers are active *in vivo* and successfully disrupt the function of their target. The anti-CDK2 aptamer causes cell-cycle arrest, the anti-E2F aptamer inhibits cell proliferation, and the anti-HPV E6 aptamer causes apoptosis in HPV-positive cancer cells [71–73].

Recently, peptide aptamers have been created containing signals that alter either the subcellular localization or ubiquitination state of their targets [74] (Fig. 5). Aptamers against CDK2 and Ste5 engineered with a nuclear localization signal, translocate their binding partners into the nucleus (Fig. 4). These transporters also blocked the activity of their target protein, which was probably caused, in part, by their altered localization [74]. An NES-containing aptamer against NFκB could re-localize NFκB from the nucleus and sequester it in the cytoplasm, thereby preventing its function as a transcriptional activator. Similarly, designing aptamers that target p53 or Forkhead transcription factors to the nucleus, while maintaining or even activating transcriptional activity, represent potential therapeutic methods.

Future implications

Using small molecules or peptide aptamers to alter the cellular localization of a protein can reveal much about its cell biology. In addition, the spatial localization of a target offers another dimension or variable that researchers could exploit to control cancer cell growth. This review has described several methods for targeting nuclear transport,

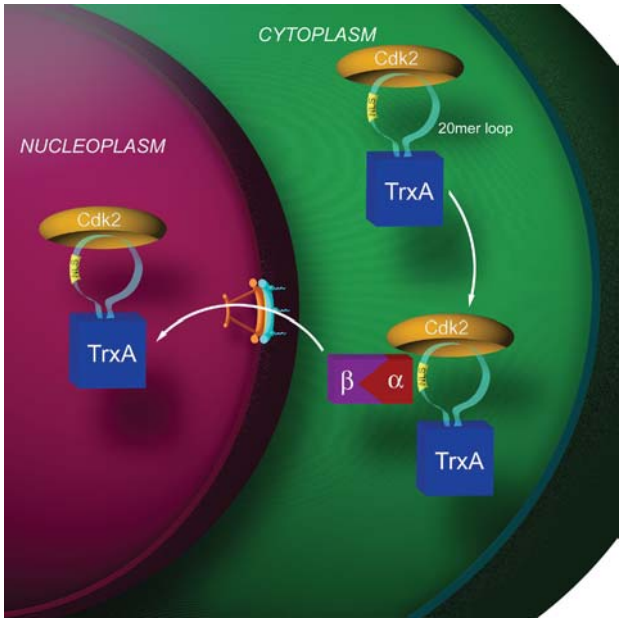

Figure 5. Peptide aptamers contain a structurally constrained region of ~20 amino acids expressed as part of an inert scaffold such as thioredoxin. Aptamers can be engineered to include a nuclear localization signal (NLS) to translocate their binding partners (i.e. Cdk2) into the nucleus using the nuclear transport machinery of the cell.

and these methods can range widely, from inhibiting the general protein transport machinery, to using small-molecule protein or lipid-kinase inhibitors to effect changes in the transport of specific target proteins.

Keeping transcriptional activators of cell death in the nucleus is a powerful way of destroying aberrantly growing cells. Recently, researchers have returned to using LMB to re-activate mutated p53 tumor suppressor, as well as to stimulate cells expressing BCR–ABL to undergo apoptosis. In clinical trials, LMB has been shown to be extremely toxic. Thus, targeting the general nuclear transport machinery might result in too much non-specificity; such an inhibitor cannot delineate between healthy and unhealthy cells. Perhaps such compounds can be administered directly to certain tissues or tumors. Nonetheless, new nuclear transport factors are continually being discovered and characterized. It is possible that some transporters might be specific to a protein cargo. In addition, the crystal structure of CRM1–LMB binding has not been solved. This structure will facilitate the design of other small molecules that specifically block export and might enable researchers to make a less-toxic export inhibitor. Surprisingly, our own chemical genetic screen for FKHR nuclear export inhibitors resulted in several electrophilic compounds that target CRM1 (T.R. Kau and P.A. Silver, unpublished results). More

studies to find inhibitors of nuclear transport, particularly protein import, will be highly beneficial and could result in potential and novel anticancer agents.

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