The nuclear import receptor Kpnβ1 and its potential as an anti-cancer therapeutic target

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Abstract

Many proteins require transport across the nuclear envelope, the physical barrier separating the nucleus from the cytoplasm. Karyopherin β proteins are the major nuclear receptor proteins in the cell that cargo proteins across the nuclear envelope, allowing them to enter and exit the cell nucleus. Karyopherin β1 (Kpnβ1), a major nuclear import receptor, plays an integral role in importing transcription factors, cell signlaing proteins, cell cycle proteins, etc. into the nucleus, thus playing a crucial role in maintaining normal cell homeostasis. However, cancer cells appear to differentially regulate the expression of the Karyopherin β proteins, presumably in order to maintain increased nuclear transport rates, thus implicating this protein family as a target for cancer therapy. The role of Kpnβ1 in cancer is only now being elucidated, and recent work points to its potential usefulness as an anti-cancer target.

Introduction

Access to the nucleus is limited by the nuclear envelope, a double-membrane structure in which large pore structures named nuclear pore complexes (NPC) are embedded. Molecules smaller than approximately 20-30 kDa can passively diffuse through the NPC, however, larger macromolecules generally require active receptor-mediated transport for entry/exit into the nucleus. The Karyopherin superfamily of proteins are soluble nuclear transport receptor proteins that are involved in the shuttling of cargo proteins, and certain RNAs, across the nuclear pore complex (NPC) into and out of the cell nucleus. The channel of the NPC through which Karyopherin-mediated transport occurs appears to be flexible and can

expand to allow for the transport of large cargoes. Each NPC is composed of up to around 100 different proteins, called nucleoporins (Nups) (1). The majority of Nups contain tandemly repeated phenylalanine-glycine (FG) motifs that line the central channel of the NPC. These are the motifs that Karyopherin proteins bind as they transport their cargoes through the pore.

The Karyopherin protein family is divided into alpha and beta subfamilies, with almost all known nuclear import and export pathways being mediated by Karyopherin β family members, while the Karyopherin α family members are described as adaptor proteins (2). Although not all proteins needing to enter or exit the nucleus will utilise the Karyopherin proteins for transport, for example β-catenin translocates across the NPC on its own (3), a vast proportion of proteins within the cell depend on Karyopherin protein function, highlighting the importance of this protein family in maintaining correct cell functioning.

This review focuses on recent work that highlights the role of Karyopherin β proteins in cancer. We describe Karyopherin β-mediated transport, with specific focus on the association between Karyopherin β protein expression and cancer, and new inhibitors of the Karyopherin β proteins, specifically Kpnβ1.

Karyopherin β proteins as mediators of nuclear transport

In mammalian cells there are more than twenty Karyopherin β family members. While some have been well characterised, the function of others is still unknown. Nine have been shown to act as export receptors (exportins), binding their cargo in the nucleus and transporting it through the NPC into the cytoplasm. Crm1 (the chromosome region maintenance 1 protein) is the major export receptor, also known as Exportin 1 (Xpo1). Crm1 recognises proteins containing a nuclear export signal (NES). Other Karyopherin β members are known to function as import receptors (importins); these proteins bind their cargo in the cytoplasm and transport it through the NPC into the nucleus. An import function has been demonstrated for eleven Karyopherin β family members. Although nuclear transport mediated by members of the Karyopherin β protein family is thought to occur by essentially similar mechanisms, the different receptors do appear to have distinct functions. Transportin 1, as an example, mediates the import of mRNA-binding proteins, while Importin 5 and Importin 7 mediate the import of ribosomal proteins (4). Moreover, some of the Karyopherin β members, such as Importin 13 and Exportin 4, are able to function as both import and export receptors. While diverse, there are certain features that characterise the Karyopherin β proteins; these include an N-terminal RanGTP-binding motif (RanGTP binding provides the energy required for Karyopherin β-mediated transport), a large size of between 90 and 130 kDa, an acidic isoelectric point of between 4.6 and 5.9, and an ability to interact directly with the nuclear pore complex (1;4).

Karyopherin β1 (Kpnβ1), a major nuclear import receptor

Kpnβ1 (also known as importin β, p97, or yeast Kap95) was the first identified nuclear transport factor and is found at a concentration of approximately 3 μM in the cell (5). It is

the major import receptor (importin protein) in the cell and can function in combination with up to 11 different partners to ferry distinct cargoes (2). Karyopherin α family members (Kpnα1-6) commonly act as adaptor proteins and work in concert with Kpnβ1 to transport cargo proteins into the nucleus, although Kpnβ1 has also been shown to partner with other Karyopherin β-like receptors. Each Kpnβ1 heterodimer has distinct cargo specificities and the particular recognition signal on the cargo protein, as well as the general protein context in the cell, specify which adaptor protein should bind (6;7). While Kpnβ1 commonly transports cargoes in concert with an adaptor, it can transport certain cargoes on its own; examples of this include transport of the parathyroid hormone-related protein (PTHrP) (8) and the sterol regulatory element binding protein 2 (SREBP-2) (9). The structure of Kpnβ1 allows for its interaction with its many binding partners and cargoes, whereby the protein is coiled into a short superhelix, with extensive interaction surfaces both on the outside and the inside of the short superhelix (2). The Kpnβ1 superhelix is comprised of 19 helical repeats, termed HEAT domains, each of which consists of two alpha helices joined by a loop.

Generally, Kpnβ1 cargo proteins contain a specific recognition signal sequence or nuclear localisation signal (NLS) in their amino acid sequence. The first identified NLS was that of the SV40 large T-antigen (10), which consists of a short stretch of basic amino acids, designated as the basic type or “classical” NLS (cNLS). This type of NLS is divided into two groups, monopartite and bipartite (11). Monopartite NLSs contain a single cluster of basic lysine and arginine amino acids (PKKKRKV) (P: proline; K: lysine; R: arginine; V: valine), while bipartite sequences contain two clusters of basic residues separated by an unconserved linker region ($KRX_{(10\text{-}12)}KRRK$) (11). The classical NLS-containing proteins are recognised by Kpnα, which

subsequently binds Kpnβ1, through its importin β-binding domain (IBB), and classical nuclear import occurs (12). Not all import cargoes, however, contain a cNLS and many non-classical NLS motifs exist that do not require an adaptor protein like Kpnα for nuclear import, but rely on Kpnβ1 directly, or alternatively one of the other Karyopherin β members.

Nuclear transport via Karyopherin β1 requires the Ran-GTP nuclear transport cycle

Kpnβ1-mediated nuclear transport requires energy provided by Ran, a small Ras-like guanosine triphosphatase (GTPase). In the classical nuclear import pathway, the cargo protein is recognised by Kpnα in the cytoplasm, becomes bound by Kpnβ1, and localises to the nuclear envelope. After translocation through the nuclear pore complex, RanGTP binds, inducing the allosteric destabilisation and release of the import cargo and Kpnα. Kpnα is recycled back to the cytoplasm via its own nuclear exporter, the cellular apoptosis susceptibility protein, Cas (Cse1L). Kpnβ1 is separately transported back to the cytoplasm, bound by RanGTP. Back in the cytoplasm, RanGTP is hydrolysed to RanGDP and Kpnβ1 is released for its next cycle. RanGDP is transported back into the nucleus via its import factor, Ntf2 (13). Ran effectors are responsible for the GDP/GTP-state that Ran is in. Ran in the cytoplasm becomes bound by GDP due to the action of the Ran GTPase activating protein (RanGAP), as well as the Ran-Binding Protein 1 (RanBP1), localised in the cytoplasm. Ran in the nucleus, however, is in a GTP-bound state due to the RanGTP exchange factor (RanGEF or RCC1) that is bound to chromatin in the nucleus.

The structure of Kpnβ1 supports its function, where HEAT repeats 1-8 (or residues 1-364) of the Kpnβ1 protein are required for the binding of Kpnβ1 to RanGTP, while the importin-β-binding (IBB) domain of Kpnα interacts predominantly with residues 331-876 located within HEAT repeats 7-19 of Kpnβ1 (5). Thus, RanGTP-mediated release of Kpnα from Kpnβ1 is likely to be an active displacement rather than due to simple competition between Ran and Kpnα for a common binding site. In addition, Kpnβ1 has at least two non-overlapping sites of interaction with the NPC (FG repeat binding sites), which could potentially be used sequentially during translocation (5). These are located between residues 152 and 352, corresponding to HEAT repeats 4-8 (14). It has been proposed that the two FG repeat binding sites could allow Kpnβ1 to bind to bind two nuclear pore proteins at once, binding one and releasing the other as part of a multistep translocation through the nuclear pore (15).

Kpnβ1 cargo molecules

Kpnβ1 imports numerous proteins that require entry into the nucleus. Multiple transcription factors are transported by Kpnβ1, for example NFκB p65 (16) and Stat3 (17). These proteins are translated in the cytoplasm but are only active once in the nucleus and so depend on import into the nucleus following their translation for their function of binding and activation or repression of the promoters of target genes. Moreover, many cell signalling networks are highly dependent on the timely translocation of proteins from the cytoplasm into the nucleus. Extracellular signals bind to cell surface receptors, initiating cascades of protein phosphorylation and dephosphorylation events, leading to the nuclear translocation of various kinases, phosphatases, or transcription factors. Many of such signalling networks

depend on Kpnβ1 protein function, for example the MAPK pathway, where phosphoERK enters the nucleus via Kpnβ1 (18). In addition, cell cycle proteins are shuttled into or out of the nucleus when the appropriate signals are given (19). Kpnβ1 thus plays a crucial role in the correct expression of genes, in cell signalling cascades, and in the control of the cell cycle, and in this way impacts many of the integral processes in the cell.

Additional Kpnβ1 functions

In addition to its nuclear transport functions, Kpnβ1 is also an important regulator of mitosis, contributing to spindle assembly. After the breakdown of the nuclear envelope in mitosis, it has been shown that Kpnβ1 regulates spindle assembly by binding to and sequestering key spindle assembly factors (SAFs), impairing their microtubule-stabilising or –organising activities (20). However, Kpnβ1 is released from the SAFs in areas where RanGTP is present. RanGTP is produced only in the region of the mitotic chromosomes because the RanGEF RCC1 remains chromosome-bound at mitosis. Thus, near the mitotic chromosomes, RanGTP releases Kpnβ1 from the SAF spindle assembly factors, allowing it to promote localised spindle formation.

Kpnβ1 has also been implicated in a variety of other cellular processes including postmitotic nuclear envelope assembly and nuclear pore complex assembly (21). In addition, Kpnβ1 has been implicated as a cytoplasmic chaperone, whereby it shields the basic domains of certain proteins (for example ribosomal proteins, histones and other basic nuclear proteins) preventing their aggregation with polyanions in the cytoplasm after translation (2). A

specific role has been shown for the Kpnβ1/Imp7 heterodimer in suppressing the aggregation of histone H1 (22).

Regulation of Kpnβ1 expression

Since Kpnβ1 is involved in multiple nuclear import pathways, as well as having several other non-import functions, of which new functions are likely to yet be elucidated, Kpnβ1 literally impacts thousands of proteins and is crucial for the maintenance of correct cellular physiology (2). As normal cell homeostasis depends on the integrity of Kpnβ1 function, its tight regulation and correct expression is very important. To date there are no known disease-causing mutations in Kpnβ1, or any other components of the nuclear transport machinery (23). The absence of mutations and chromosomal translocations underscores the essential nature of Kpnβ1 even at the single cell level.

Little is known regarding the regulation of Kpnβ1 expression, although Kpnβ1 expression has been shown to be responsive to stress, including heat shock, ethanol and oxidative stress (24). In addition, evidence has suggested that Kpnβ1 expression is controlled in a cell cycle-dependent manner (in the yeast *Saccharomyces cerevisiae*) (25), likely impacting nuclear import rates during the cell cycle (26). A recent study by van der Watt et al. (2009) showed that the Kpnβ1 promoter is regulated by the direct binding of the E2F transcription factor, a well known S-phase regulator, to three specific sites located in its promoter, verifying its cell cycle-dependent regulation (27). Moreover, in an *in silico* study performed by Quan et al. (2008), it was suggested that most Karyopherin β proteins, including Kpnβ1, are regulated by Sp1, NFY, NRF-2 and RREB-1 transcription factors, which are all known to play important

roles in the cell cycle (26). Expression of Crm1, the major nuclear export receptor, has indeed been shown to be regulated by the binding of Sp1 and NFY transcription factors to its promoter (28).

The cell cycle regulation of Kpnβ1 suggests that its expression may associate with proliferation. Indeed Kpnβ1, along with twelve other Karyopherin β genes, shows comparatively high expression in tissues that proliferate actively, for example lymphocytes, tumours, testis and stem cells (23). Quan et al. (2008) state "the functional importance of β-karyopherins determines their expression" (26). Hence it is likely that Kpnβ1 expression is increased where there is an increased requirement for nuclear import. In line with this, it has been shown that the rate of nuclear import can be modulated at least 10-fold by the concentration of Kpnβ1 in the cell (29).

An association between nuclear transport proteins and cancer

Cancer cells display altered nuclear transport. It is reported that as cells pass from quiescence to proliferating to transformed, the rates of nuclear transport increase (30;31). It is likely that nuclear transport rates are increased in transformed and cancer cells in order to sustain their increased metabolic and proliferative abilities. Kuusisto et al. (2011) recently reported that in order to increase nuclear transport rates, transformed cells display increased levels of certain Karyopherin β proteins (32). In line with this, over the last few years several lines of evidence have emerged supporting the idea that the increased nuclear transport rates in cancer are due, in part, to increased Karyopherin β protein expression. For

example, in several cancer cell lines and human tumours, the cellular apoptosis susceptibility protein (Cas or Cse1L), a Karyopherin β protein that mediates the nuclear to cytoplasmic translocation of Kpnα, is found overexpressed (33-38). This upregulation triggers the nuclear accumulation of Kpnα-dependent transcription factors, thereby mediating increased cell proliferation. In addition, high Crm1 expression has also recently been linked to cancer. Its overexpression has been reported in cervical cancer (39), ovarian cancer (40), osteosarcoma (41), glioma (42) and pancreatic cancer (43), with high levels of Crm1 being found to associate with poor patient survival. Similarly, overexpression of the Kpnβ1 adaptor protein, Kpnα2, has been reported in melanoma (44), breast cancer (45;46), cervical cancer (39), oesophageal cancer (47), ovarian cancer(48), non-small cell lung cancer (49), prostate cancer (50), bladder cancer (51) and liver cancer (52), and found to correlate with a shorter disease-free survival.

There are limited studies that describe the overexpression of Kpnβ1 in cancer, although van der Watt et al. (2009) showed that Kpnβ1 is expressed at elevated levels in cervical tumour tissue and cell lines compared to normal cervical epithelium (39), and Smith et al. (2010) found that Kpnβ1 mRNA was elevated in ovarian cancer cell lines and transformed ovarian cells compared to normal primary ovarian epithelial cells (and to a certain extent in breast cancer cells compared to normal breast epithelial cells) (18). Furthermore, Kuusisto et al. (2011) described increased levels of Kpnβ1 in several transformed cell lines, compared to their respective untransformed counterparts (32), suggesting that Kpnβ1 upregulation is indeed associated with cellular transformation and cancer.

As the nuclear transport cycle employed by Karyopherin β proteins often requires energy provided by Ran, it follows that for increased Karyopherin β protein expression to translate into increased nuclear transport rates, Ran expression also needs to be elevated in cancer cells. This is in fact the case, as increased expression of Ran has been reported in various cancer types, with the cancer cell reported to be reliant on high levels of Ran expression for survival (53).

Based on these literature reports, we propose a model, shown in Figure 1, where increased expression of certain Karyopherin β proteins in cancer cells results in increased nuclear transport efficiency, thus facilitating increased oncogenic signalling and promoting the cancer phenotype.

Inhibition of Karyopherin β proteins as a cancer therapy

Since altered Karyopherin β protein expression and function has been shown to play a role in cancer development, the inhibition of Karyopherin β proteins could be an important anti-cancer strategy. However, drugs targeting these proteins are currently limited. Leptomycin B (LMB, also known as CI-940 or elactocin), a small molecule inhibitor of Crm1 (54), is an unsaturated branched-chain fatty acid that alkylates a single cysteine residue on the Crm1 protein (55), thereby preventing Crm1 from binding to and transporting its cargo proteins. It was shown to exhibit anti-cancer activity *in vitro* and *in vivo* (56) and was tested as a potential cancer therapeutic against a range of tumours in phase I clinical trials (57), although exhibited high toxicity when administered systemically, and so was discontinued

due to the associated side-effects (57). It is not clear whether these side-effects were due to off-target toxicity and many maintain that targeting Crm1 could still prove to be a novel and potent anti-cancer therapy, perhaps in lower concentrations or in combination with other therapies (58). Alternatively, a Crm1 inhibitor might be better tolerated if administered as a local rather than systemic therapy (59). Ultimately, LMB has established a model for inhibition of Crm1, and has also proved to be extremely useful for the analysis of nucleocytoplasmic transport of hundreds of endogenous, as well as viral proteins. LMB also acts as a reference point from which novel inhibitors can be designed. Mutka et al. (2009) has recently published a report on a series of new nuclear export inhibitors (NEIs) (60). These compounds are semisynthetic LMB derivatives that showed improved therapeutic potential compared to LMB. They maintain the high potency of LMB but are up to 16-fold better tolerated *in vivo* (60). Recent work has also reported the development of additional small molecules that target Crm1 and inhibit nuclear export, specifically N-azolylacrylates (61).

The identification of Crm1 as the protein target for LMB has spurred substantial interest in targeting the nuclear export and import machinery for drug discovery. Current work is underway in an attempt to "drug" other members of the Karyopherin β protein family, as well as to innovate creative ways of exploiting the nuclear transport process to improve the efficacy of current treatment options. For example, Aronov et al. (2004) describe the conjugation of anticancer agents, such as carboplatin analogues, to a poly(ethylene glycol) carrier and an NLS resulting in a rapid internalisation of the drug and an efficient accumulation in the nucleus (62). Although underused today, use of an NLS to direct drugs

to the nucleus may be a novel and efficient way to improve drug efficacy in the future (63). Another way in which the nuclear transport process could be exploited in order to improve the sensitivity of cancer cells to chemotherapeutic agents is by co-treating with a nuclear import inhibitor that can prevent the nuclear accumulation of proteins like NFκB and EGFR, whose respective nuclear accumulations are known to contribute to drug resistance following chemotherapy (64;65).

Kpnβ1 as a target for cancer therapy?

The increased expression and functional reliance on Kpnβ1 in transformed and cancer cells suggests that targeting Kpnβ1 itself may be an attractive approach to treating cancer. However, since both normal and cancer cells share the same nuclear transport machinery, there are concerns that its inhibition might induce side effects by inhibiting the proliferation of normal tissues. While this concern is valid, there is evidence that targeting proteins that normal cells require but on which cancer cells have increased dependence is a feasible cancer treatment option. An example is the targeting of Myc, a transcription factor required for the growth of somatic cells, yet to which cancer cells are "addicted" as it co-ordinates vital oncogenic signals (66). Soucek et al. (2008) showed that the inhibition of Myc *in vivo* triggered the rapid regression of lung tumours, and although it exerted effects on normal tissues, these effects were well tolerated and rapidly and completely reversible (67). Similar results have been obtained in other cancer types (66).

Following on from the success of targeting Myc in *in vivo* model systems, the targeting of Kpnβ1 could have therapeutic benefits. This is evidenced in part by a study by van der Watt et al. (2009), which shows that the inhibition of Kpnβ1 (using siRNA) in cancer and transformed cells leads to cell death via apoptosis, yet Kpnβ1 inhibition in normal cells has only a minor effect on cell viability (39). Thus, the feasibility of targeting Kpnβ1 as an anti-cancer therapeutic strategy needs to be evaluated. However, the limitation currently lies in the lack of availability of Kpnβ1 inhibitors, or actually of nuclear import inhibitors in general. Up until a few years ago, the only available inhibitor of nuclear import was the lectin, wheat germ agglutinin (WGA) (68). WGA functions by blocking nuclear pore complexes, thus preventing nuclear import from occurring (69). Recently, however, a few nuclear import inhibitors with more specific targeting functions have been identified. These include two peptide inhibitors, bimax1 and bimax2, that were recently found to bind tightly to Karyopherin α independently of Kpnβ1, preventing the release of the cargo in the nucleus (70); and a peptide designated M9M, which was found to bind Karyopherin β2 (transportin), inhibiting its nuclear import function (71). Small molecule peptidomimetic inhibitors of Karyopherin α/β have also been described and used to study nuclear import *in vivo* (72), however, these inhibitors are not cell-permeable. Ivermectin is a broad-spectrum anti-parasitic recently found to inhibit Karyopherin α/β, however, does not appear to block import mediated by Kpnβ1 alone (73). Finally, Karyostatin 1A is the first novel small molecule inhibitor of Kpnβ1 to be identified; however, its off-target effects have not yet been examined (74). In addition, it has not yet been determined whether Karyostatin 1A exhibits any anti-cancer effects. Overall, drugs targeting Kpnβ1-mediated nuclear import are very limited, and much work remains to be done in order to determine the potential of Kpnβ1, and its protein family in general, as a target for cancer therapy.

Conclusions and Perspectives

The extensive roles played by Kpnβ1 implicate it as a global regulator of the cell and essential for cell functioning. Yet, its deregulation in transformed and cancer cells and the associated increased reliance on its expression suggest that it has potential as a novel target for cancer therapy. Further research is required to fully elucidate the potential clinical usefulness of Kpnβ1, and its other Karyopherin β family members, as anti-cancer therapeutic targets.

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Figure legend

Figure 1. A diagrammatic representation of Karyopherin β-mediated nuclear transport in normal (a) and cancer (b) cells. a). Under normal conditions, nuclear import and export occurs predominantly via the Karyopherin β proteins, Kpnβ1 and Crm1, respectively, where Kpnβ1 can bind its cargoes directly, or in complex with its adaptor, Kpnα. b). In cancer cells, expression of certain Karyopherin β proteins (including Kpnβ1 and Crm1) is

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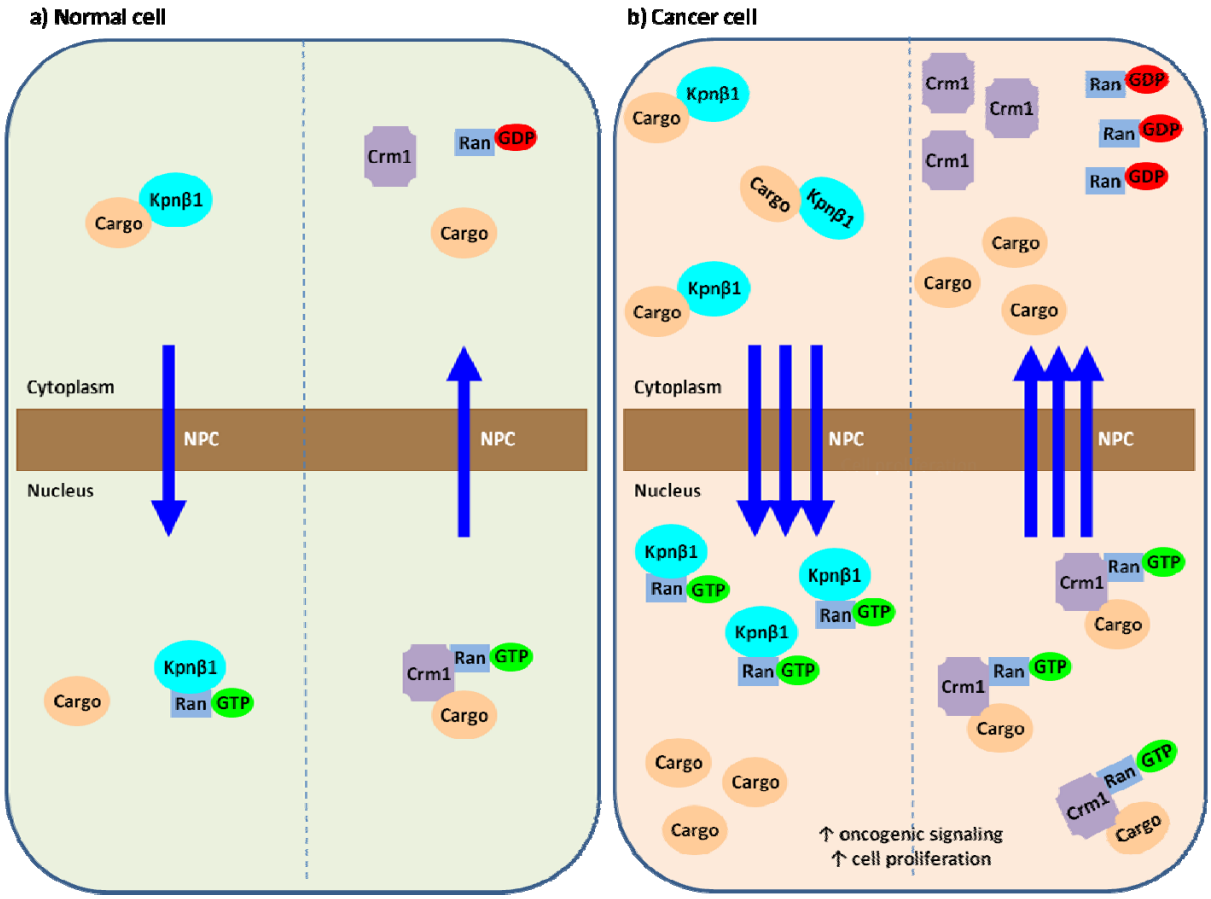
Figure 1


a) Normal cell
b) Cancer cell
Kpnβ1
Cargo
Crm1
Ran
GDP
GTP
Cytoplasm
NPC
Nucleus
↑ oncogenic signaling
↑ cell proliferation