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Diversification of importin-α isoforms in cellular trafficking and disease states

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Abstract

The human genome encodes seven isoforms of importin α which are grouped into three subfamilies known as α1, α2 and α3. All isoforms share a fundamentally conserved architecture that consists of an N-terminal, autoinhibitory, importin-β-binding (IBB) domain and a C-terminal Arm (Armadillo)-core that associates with nuclear localization signal (NLS) cargoes. Despite striking similarity in amino acid sequence and 3D structure, importin-α isoforms display remarkable substrate specificity *in vivo*. In the present review, we look at key differences among importin-α isoforms and provide a comprehensive inventory of known viral and cellular cargoes that have been shown to associate preferentially with specific isoforms. We illustrate how the diversification of the adaptor importin α into seven isoforms expands the dynamic range and regulatory control of nucleocytoplasmic transport, offering unexpected opportunities for pharmacological intervention. The emerging view of importin α is that of a key signalling molecule, with isoforms that confer preferential nuclear entry and spatiotemporal specificity on viral and cellular cargoes directly linked to human diseases.

Keywords

nuclear transport; importin-α isoforms; importin α1; importin α3; importin α5; importin α7; IBB domain

INTRODUCTION

Nucleocytoplasmic transport is central to the function of eukaryotic cells and an integral part of the processes that lead to many human diseases. Over the past 30 years, dramatic progress in cell, molecular and structural biology has been instrumental in elucidating the soluble factors mediating the trafficking of cargoes through the nuclear pore complex (NPC), the organization, architecture and assembly of the NPC, and the mechanisms of translocation through it. The overall picture emerging from this large body of work describes nucleocytoplasmic transport as a signal- and energy-mediated process through the large aqueous channel of the NPC, which functions as a semipermeable filter. The importin β-superfamily, named for the founder importin β (also known as karyopherin β1) [1],

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represents the major class of soluble transport receptors involved in shuttling cargoes through the NPC. Importin β functions as a transport receptor by coordinating three biochemical activities: (i) high-affinity binding to a basic nuclear localization signal (NLS) exposed by import cargoes; (ii) avidity for phenylalanine–glycine repeats exposed by several nucleoporins (FG-nups) protruding into the cytoplasm and lining the NPC; and (iii) high-affinity association with Ras-related nuclear protein GTP (RanGTP) which triggers a conformational change to release both NLS-cargoes and FG-nups. Importin β can associate with import cargoes either directly or via adaptors, such as snurportin or importin α [2]. These adaptors can be thought of as specialized cargoes that carry a potent N-terminal NLS, known as the importin β-binding (IBB) domain [3].

Import cargoes expose NLS sequences, exemplified by the monopartite SV40 large T-antigen NLS and the bipartite nucleoplasmin NLS [4], which bind the adapter importin α in the presence of importin β, initiating what is known as the ‘classic’ nuclear import pathway (Figure 1). The heterotrimeric importin β/α/NLS-cargo complex (Figure 2) shuttles through the NPC and delivers the NLS-cargo into the nucleus in a process aided by the small RanGTPase. Although a detailed description of nuclear transport is beyond the scope of the present review, several excellent reviews describing mechanisms governing nucleocytoplasmic transport have been published in recent years [5–13]. In the present review, wefocus on a surprising aspect of the biology of importin α: unlike importin β, which is encoded by a single gene in all eukaryotes, at least seven isoforms of importin α exist in higher eukaryotes. These isoforms display remarkable substrate specificity *in vivo*, which is not always repeated *in vitro*. The present review focuses on the biology of importin α isoforms: we take an inventory of known NLS-cargoes specific to importin α isoforms and critically analyse their role in cell physiology and involvement in human diseases.

EVOLUTION AND DIVERSIFICATION OF THE IMPORTIN-α GENE IN EUKARYOTES

Functional diversification of the adaptor importin α has occurred throughout the evolution of multicellular animals, paralleling the increasingly complex requirements of higher organisms with their need to perform cell- and tissue-specific functions during development and differentiation [14]. *Saccaromyces cerevisiae* has a single gene encoding orthologues of importin β and importin α (known as Kap95 and Kap60, respectively). Three importin-α isoforms exist in *Drosophila melanogaster* and as many as seven isoforms are found in vertebrates (Figure 3). Human importin-α isoforms are well conserved, with 26% identity and 42% conservation in their amino acid sequences, as determined by ClustalW alignment [15] and the BLOSUM62 similarity matrix [16]. They can be divided into three subfamilies: the α1 subfamily containing importin α1 and α8; the α2 subfamily containing importin α3 and α4; and the α3 subfamily, containing importin α5, α6 and α7 (Table 1). A growing number of cellular (Table 2) and viral (Table 3) cargoes rely on specific importin-α isoforms for transport into the nucleus, and important differences in the regulation of these isoforms are just beginning to be understood.

The importin-α1 subfamily

The α1 subfamily is the least conserved of the importin-α subfamilies, with 55% identity and 71% conservation. Human importin α1 was first discovered almost two decades ago [17] and named hSRP1α based on its similarity to the yeast Kap60, also known as serine-rich protein 1 (SRP1), a suppressor of RNA polymerase 1 [18]. Another of importin α1's alternative names (see Table 1), Rch1 (or Rag cohort 1), originates from one of its first determined binding partners, RAG-1 [19]. At the same time, a similar protein, with 44 % of its sequence identical to SRP1 and 76% to human importin α1, was also identified in *Xenopus* eggs [20]. Since then, importin α1 has been considered to be the general importer of cargoes bearing a classic NLS [21] whereas its mouse homologue, importin α2 (99.2% similar to the human importin α1), has been extensively used for structural studies, both alone [22] and complexed with a variety of different NLS-cargoes [23–35]. A second member of the α1 subfamily, importin α8, was recently identified [36], although a homologue had previously been characterized in bovine cells [37]. Due to its recent discovery, little is known about this isoform apart from the fact that it is overexpressed in some forms of prostate cancer [38] and it appears to play a role in development [37].

The importin-α2 subfamily

Both members of the α2 subfamily, importin α3 and α4, were discovered in 1997, soon after importin α1 [39–42]. The two isoforms are extremely homologous, with 86% identity and 92% sequence conservation. The isoforms of this family are best known for their specificity for important cargoes such as the transcription factor NF-κBor nuclear factor κ-light-chain-enhancer of activated B cells (p50/p65) [43] and regulator of chromosome condensation (RCC)-1, Ran's only known guanine nucleotide exchange factor [21] (see Table 2). The crystal structure of importin α3 was recently determined when complexed with the influenza A virus polymerase subunit PB2 [35] (see Table 1), whereas the structure of importin α4, arguably very similar to α3, is unknown.

The importin-α3 subfamily

The α3 subfamily has the highest homology to yeast Kap60 and is thought to have branched off earlier in evolution than the other subfamilies [14] (see Figure 3). Importin α5 was discovered about the same time as importin α1, and was initially named NPI-1 based on its interaction with influenza nucleoprotein (NP) [44,45]. Importin α6 [41] and α7 [21] were discovered soon afterwards, but, due to high homology within the family (74% identity and 82% sequence conservation), functional differences between the isoforms have not been determined. However, the most striking difference within this family is the restriction of importin-α6 expression to the testis [41]. The best characterized cargoes specific to this family are the phosphorylated STAT1 (signal transducer and activator of transcription 1) homodimer or STAT1/STAT2 heterodimer [46,47] (see Table 2). These specialized dimeric cargoes contain a non-classic import signal which, unlike classic NLS-cargoes, can import only in the context of STAT1 [48,49] and appears to rely on contacts with the C-terminus of importin α5 [49]. The structure of importin α5 was determined in two different conformations, with influenza A PB2 [50] and the nucleoporin Nup50 [51]; importin α7 was

also resolved when complexed with PB2 [35], and a fragment of importin α6 spanning armadillo (Arm) 7–10 was recently found bound to Ebola virus VP24 [52] (see Table 1).

Spatiotemporal distribution of importin-α isoforms

All importin-α isoforms are expressed to some extent in all adult cell types [21,41,53], with the exception of importin α6, which is limited to the testis [41], and importin α8, the expression of which is restricted to the ovaries and early stage embryos [37,54]. However, the relative intracellular concentration of isoforms α1, α3, α4, α5 and α7 has not been accurately determined, and it is not known if any particular isoform is generally abundant. Furthermore, it is increasingly appreciated that the distribution of importin-α isoforms across different cell types and at different developmental stages is important in development and normal cellular functions, e.g. mouse neural differentiation displays a specific expression sequence of importin α: a gradient from high initial importin α1 and low importin α3 and α5, to lower importin α1 and high importin α3 and α5 as differentiation progresses [55]. An additional layer of import regulation in neural differentiation comes from the evidence that certain cargoes such as Oct6 or Brn2 can bind to multiple importin-α isoforms, but their transport outcome varies depending on the isoform. Binding to importin α5 leads to successful nuclear import, whereas binding to importin α1 inhibits cargo import, causing cytoplasmic retention [56]. Notably, these cargoes bind an importin-α1 C-terminal-binding section (iCBS), possibly in a similar fashion to Nup50 [57, 58].

The exact mechanism by which importin α1 prevents nuclear import is unknown, but two possible scenarios are either difficulty in complex disassembly or conformational change in the import complex which prevents its import [56]. An examination of importin-α isoform expression in different regions of the mouse brain, at various stages after birth, also shows interesting changes in distribution over time and regions of the brain [59]. Likewise, importin-α isoform expression in spermatogenesis has been extensively studied, showing significant variations in expression levels of importin α1, in particular, throughout the process [58,60,61]. Any one of the importin α4, α5, α7 or α8 knockouts in mice did not lead to abnormal brain development [54,62–65], suggesting that the loss of a single isoform gene is probably compensated for *in vivo* by an isoform of the same subfamily. Instead, the importin α5 and α7 knockouts led to reproductive problems in female mice and the importin α8 knockout causes reduced fertility and sex imbalance in litters due to induced lethality in females [54], suggesting some non-redundant function of these isoforms in reproduction. There have been several recent studies using a proteomic approach to understand cargo specificity in spermatogenesis and development, although they have generally been looking at cargoes only in the context of one or two isoforms rather than the full range [58,66,67].

DISTINCTIVE PROPERTIES OF IMPORTIN-α ISOFORMS

All importin-α isoforms share a fundamentally conserved structure consisting of an N-terminal, autoinhibitory, IBB domain spanning approximately the first 70 residues and a C-terminal helical core containing Armadillo repeats (Arm-core) (see Figure 2). Despite the high sequence similarity, importin-α isoforms present subtle differences in 3D structure and biochemical properties which contribute to their *in vivo* specificity for specialized import cargoes.

Structure of the Arm-core

The central core of all importin-α isoforms is built by 10 stacked Arm repeats [Figure 4A (i)] and structurally very similar to Kap60 [68]. Secondary structure matching of the four human importin-α isoforms found crystallographically (α1, α3, α5 and α7) (see Table 1) with Kap60 yields an RMSD of between 0.8 and 1.7 Å (1 Å = 0.1 nm), underlining a remarkable degree of conservation between the yeast and human proteins. The Arm repeats [Figure 4A (ii)] of importin α are very similar to the HEAT repeats [Figure 4A (iv)] found in importin β; in both proteins, stacks of Arm/HEAT repeats generate superhelical solenoids, which are highly specialized to participate in protein– protein interactions [1,12]. HEAT repeats are composed of two α-helices (named H1 and H2) arranged as a hairpin, which allows for extremely flexible assemblies [69]. In contrast, in Arm repeats the first helix splits into two helices to make a total of three helices (named H1, H2 and H3), which have a triangular arrangement, leading to more rigid assemblies [70,71]. In all importin-α isoforms, two Arm repeats deviate from the standard three-helix architecture: Arm 1, which lacks the first helix (H1) [Figure 4A (iii)] and Arm 5 which has helices H1 and H2 fused together, making it essentially a HEAT repeat [Figure 4A (iv)]. Stacking of Arm repeats generates an extended concave surface, which harbours NLS-binding pockets, and a convex surface. Superimposition of the Arm-core of importin α1, α3, α5 and α7 reveals that the NLS-binding surface is practically identical in all the isoforms [35], whereas the convex surface is quite variable (Figure 4B). This implies that differences in import cargo specificity and binding affinity among importin α isoforms cannot be ascribed to the NLS-binding surface, which is fundamentally identical in all of the *a* isoforms.

The Arm-core of importin α has generally been considered to be essentially rigid compared with flexible HEAT-containing proteins such as importin β [71,72]. However, recent molecular dynamic simulations comparing the flexibility of mouse importin α1, and human importins α3 and α7, revealed that importin α3 has a greater range of extension and compression than the other isoforms, potentially due to a hinge at the major NLS-binding site [35]. This flexibility may result in lower binding affinity for classic NLSs at the major NLS-binding pocket and contribute to decreased IBB domain autoinhibition. In direct relation to the flexibility of Arm-core, the initial structure of importin α5, determined when complexed with PB2, had a domain swap of Arm 10's helix H3 between adjacent molecules in the crystal [50], although two subsequent structures found with this isoform show Arm-10 helices neatly stacked (Protein Databank PDBs 3TJ3 and 4B18) [51]. Although the physiological significance of this domain swap is unclear, Arm-10 unfolding in importin α5 may be due to the presence of a bulky Tyr^{476} at the interface of helices H1 and H2 [Figure 4B (v)]. This residue, which is conserved only in members of the α3 subfamily, but replaced by a glycine in all other isoforms, was proposed to swing inwards between helices H1 and H2, thereby preventing the intramolecular stacking essential to stabilize the folded conformation of Arm 10. Introducing a glycine/alanine at this position in isoforms α5 and α6, as in importin α1, disrupts high-affinity binding to pSTAT1 [49] and Ebola virus VP24 [52], suggesting that an intrinsic flexibility of Arm 10 could be important for binding to non-classic cargoes interacting with the C-terminus of α3-subfamily members.

Autoinhibition by the IBB domain

Similar to Kap60, the murine importin α1 was also found to be autoinhibited by its IBB domain [22]. The latter contains a bipartite basic sequence ('RRRR$(X)_{17}$KRR') and, in the absence of importin β, it folds back to occupy the NLS-binding surface of importin α (Figure 5A). The first basic motif binds at the minor NLS pocket (Arm 6–8) whereas the second basic motif occupies the major NLS-binding box (Arm 2–4) [22,73–75]. The intramolecular interaction between IBB domain and Arm-core prevents binding of importin β to an empty importin α, thus representing a regulatory mechanism to prevent futile nuclear translocation of unloaded import complexes. IBB domain autoinhibition is essential *in vivo* in yeasts, and probably in higher eukaryotes [76], and was initially thought to occur in all human importin-α isoforms. In contrast, recent studies revealed that IBB autoinhibition varies greatly among isoforms and each isoform can be differentially autoinhibited for different NLS-cargoes, e.g. both importin α3 and α5/α7 are significantly less autoinhibited than importin α1 [35]. This is probably explained by subtle differences in the IBB-domain amino acid sequence (Figure 5B): although the major NLS-binding box ('KRR') is conserved in all importin-α isoforms, the minor NLS-binding cluster shows significant differences. Importin α1, α5, α6 and α7 all have 'RRRR' as their minor NLS-binding box, whereas importin α3/α4 lacks the third arginine ('RRQR' and 'RRHR', respectively). Furthermore, in α3-subfamily isoforms (α5/α6/α7), the 'RRRR' motif is surrounded by an exceptionally acidic patch containing as many as five glutamate/aspartate residues.

The ability to overcome IBB domain autoinhibition also varies greatly among NLS sequences. Monopartite SV40-like NLSs are usually insufficient to overcome IBB autoinhibition and, thus, bind importin-α isoforms exclusively in the presence of importin β. In contrast, recent studies have shown that certain cargoes containing highly basic bipartite or certain non-classic NLSs are able to bind importin-α isoforms efficiently in the absence of importin β; noticeable examples include RCC1 [77], STAT1 [49] and influenza A virus PB2 [35]. In addition, importin α8 presents several variations in the IBB region that make it different from all other isoforms (Figure 5B). In this isoform, which has a predominant nuclear localization, the IBB domain binds with a stronger affinity to importin β and the Arm-core, but shows very weak binding to classic NLS peptides, even in the absence of its IBB domain [36]. Thus, what was once thought to be a universal feature of all isoforms of importin α, autoinhibition, appears to be generally weaker in isoforms of the subfamilies α2 and α3 and strictly cargo dependent.

IMPORTIN-α ISOFORMS IN MODULATION OF HOST–VIRUS INTERACTION

The interaction between importin-α isoforms and viral proteins has been studied since study of the nuclear transport field started, e.g. the prototypical monopartite NLS sequence (P^{126}KKKRRV^{132}), found in hundreds of cellular factors, is derived from the large T antigen of SV40 [78], and the leucine-rich nuclear export signal was first identified in the HIV Rev protein [79]. Intuitively, viruses have proteins that need to enter the nucleus to hijack the host DNA and use the cell's transcriptional machinery. Some viruses have adapted to acquire specificity for certain importin-α isoforms, although the advantage of this specialization is not well understood. Table 3 presents a comprehensive list of all viral

proteins known to interact with specific importin-α isoforms. Among them, influenza virus NP was one of the first identified NLS-cargoes to interact with importin α. Subsequent work showed that importin α1 also binds strongly and imports NP, whereas importin α3 associates only weakly with this cargo [80,81]. HIV-1 integrase, on the other hand, relies on only isoform α3 [82] and, in addition, uses a member of the β-karyopherin family, transportin, to gain access to the nucleus [83].

In a more complex example, influenza A virus polymerase subunit PB2, a major virulence determinant implicated in pathogenicity and host adaptation [84], has been shown to have a preference for importin α3 in avian influenza strains. By contrast, in mammalian-adapted strains, PB2 switches dependency to importin α7 [63,85,86], possibly as a result of a limited set of point mutations. Recent work has proposed that the preferential utilization of isoforms α3 and α7 is probably explained by the differential degree of IBB domain autoinhibition for PB2 in these two isoforms, and by the complex structure of the PB2 NLS domain, which contains a bipartite NLS next to a globular domain [35,50]. *In vitro* binding assays revealed that PB2 efficiently competes with the IBB domain of isoforms α3 and to a lesser extent α7 in the absence of importin β, whereas it is autoinhibited from binding importin α1 [35]. However, PB2 associates with comparable nanomolar affinity to the Arm-core of all isoforms in the absence of the IBB domain [87]. Another important viral protein, HIV-1 Vpr, can also bind to the C-terminal Arm repeats of importin-α isoforms from all three families [88,89], but recent studies have shown a distinct preference for importin α5. Intriguingly, although Vpr binds with similar affinity to all tested isoforms, only importin α5 efficiently released Vpr in the presence of the recycling factor CAS, and, furthermore, silencing CAS inhibited Vpr import [90]. It is unclear why CAS can induce preferential release of Vpr from importin α5, but it suggests a possible additional layer of complexity in the specificity.

In addition to promoting viral genome replication/transcription and virion assembly, another critical viral function is to evade the host's immune response. The JAK (Janus kinase)/ STAT pathway is critical in initiating the immune response to viral infections, so, unsurprisingly, it is a target for suppression by many viruses, e.g. both Ebola virus VP24 and hepatitis B polymerase block STAT1 nuclear import by binding to the C-terminus of importin α5, thereby preventing transcription of STAT1-dependent interferon genes [91,92]. VP24 has also been found to bind to other members of the α3 family [93], and a recent structure complexed with importin α6 reveals that it binds to C-terminal Arm 7–10, as opposed to the NLS-binding pockets [52]. VP24 association with importin α5 does not prevent a classic NLS-cargo from binding, suggesting that VP24 specifically blocks STAT1 import rather than all importin-α5-mediated import. However, it was also found that VP24 forms oligomers that bind to the plasma membrane [94], which could effectively sequester importin α5 in the cytoplasm and prevent any cargo relying on the α3 family shuttling through the NPC. Severe acute respiratory syndrome coronavirus (SARS-CoV) ORF6 also blocks STAT1 signalling by an indirect mechanism, via the sequestration of importin α1 on the surface of the endoplasmic reticulum [95]. Although importin α1 as such does not import STAT1, it has been hypothesized that loss of α1 increases competition for importin-α5 binding, thereby reducing the net influx of STAT1 into the nucleus. A follow-up paper

from the same group identified additional transcription factors, the transcription of which was disrupted by the ORF6 sequestration of importin α1, and some of these factors, such as cAMP response element-binding protein 1 (CREB1), SMAD4, p53 and Oct3/4, are important in the response to viral infection [96]. In addition to targeting importin α, the non-classic NLS of STAT1 is itself a target for reducing the antiviral response. A well-known example is the vaccinia virus protein VH1 [97], which dephosphorylates Tyr^{701} of STAT1 [98,99], thereby causing a conformational change that prevents exposure of STAT1's non-classic NLS to importin α5 [49], and blocks nuclear import and transcription of interferon genes.

INVOLVEMENT OF IMPORTIN-α ISOFORMS IN HUMAN DISEASES

Importin α isoforms in cancer

Importin-α isoform expression is altered in many forms of cancer. In most cases, the up-regulation of the *KPNA2* gene (encoding importin α1, see Table 1) indicates a poor prognosis, making it a useful biomarker in breast [100], ovarian [101], cervical [102], prostate [103] and bladder [104] cancer, melanoma [105], squamous cell carcinoma [106], hepatocellular carcinoma [107], lung cancer [108], astrocytic glioma [109] and anaplastic oligoastrocytoma [110]. In some cases, knock down of importin α1 with siRNA could reduce the proliferation of cancerous cells [103,108], but in others it had no effect [102]. Of particular interest was a xenograft study of epithelial ovarian carcinoma in mice, in which transplanted tumours either had *KPNA2* knocked down with siRNA, or additionally induced. It was found that knockdown of *KPNA2* reduced tumour volume, whereas increased expression of *KPNA2* increased tumour volume [111]. Other nucleocytoplasmic transporters, such as importin β [102] and CRM1 [102,110], are also found to be up-regulated in some cancers. One proposed mechanism for this up-regulation is the deregulated activity of the E2F transcription factor, which can up-regulate both *KPNA2* and the importin-β gene [112]. E2F is itself regulated by the retinoblastoma protein (Rb) pathway, which is frequently disrupted in cancer. Another importin-α isoform found to have irregular expression in cancerous cells is importin α8. Normally expressed only in oocytes and early embryos, irregular expression of importin α8 in several pancreatic cancer cell lines contributes significantly to the proliferation of these cells [38].

Importin-α isoforms in the nervous system

Specific importin-α isoforms play a role in both normal and abnormal brain and neuron function (reviewed by Perry and Fainzilber [113]). Importin α has been identified as critical in the neurons' ability to start a regenerative response in injured nerve. Importin α and β form a high-affinity complex with STAT3 that traffics retrogradely with the motor protein dynein from the axon back to the nucleus [114]. In an interesting variation on its role as a karyopherin, importin α5 was found to be critical as an adaptor to microtubules. Knock down of importin α5 in injured neurons blocked STAT3 signalling after injury, but direct injection of STAT3 into the axonal bodies, where it can be imported by other importin-α isoforms, could rescue STAT3 signalling [115], suggesting a unique role for importin α5 as a multifunctional transporter. Specific importin-α isoform expression has also been implicated in neuronal differentiation and development [55]. Analysis of the expression of

importin-α isoforms in different regions of the mouse brain between birth and adulthood shows a distinct shift in levels and ratios of importin-α isoform expression [59]. A mutation in human importin α8 has also been found to be associated with improper neuronal development [116]. However, the redundancy of the importin-α isoforms still allows for proper brain development even when one of these isoforms is lost, as seen when importin α5 was knocked down in mice [62].

Importin-α isoforms can also play a role in diseased states of the brain, e.g. in schizophrenia, importin-α3 expression is decreased, resulting in reduced NF-κB signalling. This loss of expression is associated with a single nucleotide polymorphism (SNP) in the *KPNA4* gene (encoding importin α3), which is also a marker for increased susceptibility to schizophrenia [117]. In addition, certain SNPs of the *KPNA3* gene (encoding importin α4) have been correlated with susceptibility to schizophrenia in some populations [118,119]. Importin α1 is also misregulated in Alzheimer's disease, due to it sequestration in the Hirano bodies of hippocampal neurons. The cause of this sequestration is unclear, but its result is a distinct mislocalization of critical NLS-cargoes, such as p27 [120].

Importin-α isoforms in the heart

Recent studies have shown a loss of importin-α expression in ageing myocardial tissues. In myocardial endothelial cells, a reduction in the levels of both importin α1 and importin α3 mRNA and protein was associated with reduced import of hypoxia-inducible factor 1α, which regulates vascular endothelial growth factor expression, a key component in angiogenesis. Thus, loss of importin-α expression in ageing myocardial cells could make recovery after heart disease more difficult for elderly patients [121]. In a study of human fibroblasts, the expression of importin α1 decreased steadily from children to adults to elderly people, whereas importin-β expression over this range was consistent [122]. In addition to loss of importin α1 in ageing myocardial cells, the microRNA *miR-181b* is able to reduce the signalling of NF-κB in the vascular endothelium by knocking down importin α3 expression [123]. The classic NF-κB p50/p65 heterodimer was first identified as specific to importin α3 and α4 [43], but subsequent studies have shown that it can also bind to members of the α3 family, depending on the expression system, implicating post-translational modifications [124]. This is corroborated by the observation that loss of importin α3 in the vascular endothelium blocks NF-κB signalling, but is compensated for by importin α5 in leukocytes [125].

Other pathologies

The nucleocytoplasmic shuttling of NF-κB also plays a key role in inflammatory bowel disease (IBD). Prohibitin (PHB) 1 is found to be down-regulated during IBD by tumour necrosis factor α (TNF-α), along with increased NF-κB signalling. However, when expression of PHB is restored, NF-κB signalling is reduced. Theiss et al [126] determined that expression of PHB caused suppression of importin α3 and α4, thus preventing the nuclear import of NF-κB despite cell stimulation; however, the mechanism for this suppression is unknown. Similarly, NF-κB and STAT3 signalling are found to be increased in nephropathy due to the down-regulation of *miR-223*, which causes increased expression of importin α4 and α5 [127].

REGULATION OF IMPORTIN-α ISOFORMS

An attentive review of the literature suggests at least four distinct strategies by which the availability of importin-α isoforms is controlled and regulated in response to endogenous and viral effectors (illustrated in Figure 6).

Transcriptional regulation by microRNAs

The pool of importin-α isoforms can be reduced at the transcriptional level by microRNA-mediated degradation of importin-α isoform mRNAs. Glinsky et al [128] predicted that importin α, and importin α5 in particular, is a major microRNA target in a variety of human diseases. This prediction was corroborated by subsequent studies with *miR-181b*, which was found to specifically down-regulate importin-α3 expression, thereby blocking NF-κB import and signalling in epithelial cells (Figure 6A) [123,125]. A microRNA not included in the Glinsky analysis, *miR-223*, was found to block expression of importin α4 and α5; accordingly, decreased expression of *miR-223* in nephropathy led to increased signalling of NF-κB and STAT1, possibly by expression of isoforms α4 and α5, respectively [127].

Regulation of importin-α isoform availability by sequestration

Importin-α availability can be altered post-translationally by sequestration. As previously described, Ebola VP24 binds the C-terminus of the α3-subfamily isoforms, competing for binding with STAT1 and thereby blocking its import (Figure 6B). An endogenous factor that uses a similar strategy is ARHI, a Ras homologue, which acts as a tumour suppressor and the expression of which is lost in many breast and ovarian cancers. ARHI binds several isoforms of importin α – α1, α3, α6 and α7, but not α5 – and, by doing so, it blocks import of phosphorylated STAT3 (pSTAT3) and classic NLS-cargoes [129].

Regulation of importin-α isoform concentration by targeted degradation

Another approach to reducing nucleocytoplasmic transport of expressed importin-α isoforms is targeted degradation. The porcine reproductive and respiratory syndrome virus (PRRSV) (see Table 2) can inhibit the interferon response by targeted proteasome-mediated degradation of pig importin α5. PRRSV protein Nsp1β mediates this degradation by causing an increase in ubiquitination of importin α5, although the mechanism for this is unknown [130]. Similar behaviour is seen from the foot-and-mouth-disease virus protein $3C^{pro}$ (see Table 2), which has protease activity and directly degrades importin α5 [131]. There are also endogenous approaches to targeted degradation of importin α, implemented as a response to viral infection or during cell death. Natural killer cells of the immune system are able to fight viral replication by inducing apoptosis in target cells through release of a variety of proteolytic enzymes. Among these is granzyme K, which has been found to be of particular importance as a response to influenza infection [132]. Granzyme K is a tryptase and can cleave both importin α and importin β so that they cannot bind to each other, thus blocking cargo import (Figure 6C). Interestingly, it was observed that granzyme K could cleave all human importin α isoforms at a location upstream of the IBB, at a conserved arginine residue critical for binding to importin β. This degradation has the effect of blocking viral replication by preventing the nuclear import of key influenza proteins such as NP and PB2 [133]. Importin-α cleavage has also been found to play a role in apoptosis, when various

caspases are capable of cleaving the IBB domain from importin α1, α3, α4, and α5. ΔIBB importin α could still accumulate in the nucleus, a behaviour seen previously for ΔIBB importin α from all families [134], and was found to block DNA replication by sequestering the DNA replication licensing factor (MCM) [135]. Finally, importin α5 was recently found to be a substrate for the ubiquitin ligase activity of RAG-1, perhaps as a method of regulation of RAG-1 activity [136]. RAG-1, best known for its role in V(D)J recombination, is also active as a ubiquitin ligase. Interestingly, although RAG-1 is imported into the nucleus by importin α1, not importin α5, it binds to importin α5 weakly outside its functional NLS [137].

Regulation of importin-α isoforms activity by post-translational modification

Post-translational modification of importin α and NLS-cargoes can both up- and down-regulate nuclear imports [138]. One excellent importin-α isoform-specific example is the phosphorylation of Epstein–Barr virus nuclear antigen 1 (EBNA-1). Although the unphosphorylated NLS is sufficient for nuclear import, it binds to its specific adaptor importin α5 with low affinity. Phosphorylation of the NLS accelerates the rate of import and also significantly increases binding to importin α5 [49,139]. Phosphorylation and acetylation of the importin α receptor itself have also been observed. Bannister et al. [140] found that the acetylation of importin α1 by CBP/p300 at Lys^{22}, upstream of the NLS, enhances association with importin β. CBP/p300 was also able to acetylate importin α7 but not importin α3. These authors identified a ‘GK’ motif as essential for acetylation of this importin α1, though they did not test for this in importin α7 [140]. Wang et al. [141] found that 5’-AMP-activated protein kinase (AMPK) is an upstream regulator of CBP/p300, thereby inducing importin-α acetylation and, in addition, that AMPK itself could also phosphorylate importin α1 at Ser^{105}. The combination of these two modifications significantly up-regulates human antigen R (HuR) nuclear import, whereas transfection with importin-α1 mutants lacking either modification site caused no increase in import. Acetylation at Lys^{22} is predicted to increase the affinity of the IBB domain for importin β, whereas phosphorylation of Ser^{105} is predicted to increase affinity for an NLS at the major NLS-binding pocket (Figure 6D) [141]. It is interesting to note that importin α7 has a ‘GK’ motif several residues upstream of where it is found in importin α1 (Lys^{9} compared with Lys^{22}), so it is unclear if acetylation of this isoform would have the same effect as acetylation of importin α1. Importin α3, which is not acetylated, has no’ GK’ motif in the IBB domain. In addition, no other isoform has a serine near the equivalent of importin α1’s residue 105.

CONCLUSIONS AND PERSPECTIVES

It has been 20 years since the discovery of human importin α1 [17], during which time a total of 7 isoforms and over 75 isoform-specific import cargoes have been identified (see Tables 2 and 3). As of September 2014, a Medline search for ‘importin α’ and ‘isoform’ identified 150 publications, signifying an explosion in the number of pathways recognized as mediated by specific importin-α isoforms. As described in the present review, importin-α isoforms play a pivotal role in the nuclear import of critically important transcription factors such as STATs and NF-κB, recently identified as targets of importin α5 and importin α3,

respectively. A precise display of importin-α isoform expression also seems to be critical during development, particularly of the brain. Misregulation of specific importin-α isoform expression, availability and activity occurs in disease states in response to pathogens and in cancer, which prompted many to use importin α1 as a biomarker. Thus, apart from the classic role as 'karyopherins', importin-α isoforms play a pleiotropic role as signalling molecules in cell physiology, development and human diseases.

The dilemma of *in vivo* specificity

What makes importin-α isoforms specific *in vivo*? On the basis of the literature reviewed here, we identify three factors that may help answer this question. First, in the simplest possible scenario, cell type availability of different isoforms could dictate specificity for certain import cargoes. The intracellular concentration of different isoforms has not been accurately determined, leaving open the possibility that the first discovered isoform, importin α1, may not necessarily be the most abundant in all cell types. Furthermore, as previously described, there are several mechanisms to up- or down-regulate the relative concentration of specific importin-α isoforms (i.e. microRNAs, sequestration, degradation and post-translational modifications), thereby generating fluctuations in the intracellular concentrationofimportin-α isoforms which may lead to a preferential association with certain import cargos. Secondly, not all 'import cargoes are created equal' in a cell. In addition to the NLS, other binding determinants in an import cargo can provide preferential affinity, and hence specificity, to an importin-α isoform. Good examples of this are non-classic cargoes such as STAT1 [49] and VP24 [52] which strongly bind the C-terminus of importin α, outside the minor NLS-binding pocket. Alternatively, certain NLS-cargoes such as RCC1 [77] and PB2 [35] have NLSs flanked by a folded domain that contributes additional binding determinants for importin α3. Finally, not all importin-α isoforms are equally autoinhibited by their IBB domains, suggesting that different levels of intramolecular autoinhibition may determine preferences in association (and hence specificity) of an isoform for a given import cargo. Similarly the disassembly of an import cargo from an importin-α isoform can potentially dictate isoform specificity, as in the case of Vpr. This small HIV-1-encoded protein binds to several importin-α isoforms (see Table 3), but is specific for importin α5 due to its inability to be removed from the other isoforms by CAS [90]. In all the strategies described above, post-translational modifications in both import cargoes and importin α can modulate the affinity and hence specificity of a given isoform for an import cargo [142].

Targeted inhibition of specific importin-α isoforms

A goal of this field is to develop selective inhibitors that block specific isoforms (or similar isoforms within a subfamily) without affecting the bulk of NLS-cargoes moving through the NPC. There are already examples of peptides designed to target importin α that block the classic nuclear import, such as cSN50.1, Bimax2 and ivermectin. Of these, only cSN50.1 has been shown to have importin-α isoform specificity. It is a peptide based on the NLS of the NF-κB subunit p50 [143], shown to bind with nanomolar affinity to importin α5 and only very weakly to other isoforms [144]. This peptide has been shown to block import of NF-κB along with a variety of other key transcription factors, thereby suppressing proinflammatory signals [145,146]. Bimax2, designed by Kosugi et al. [147], is a

monopartite NLS that acts as a high-affinity general inhibitor of the classic nuclear import pathway. Ivermectin is a small molecule commonly used as an anti-parasitic drug, which, it has recently been determined, blocks cargo binding to importin α [148,149]. In summary, the work done to date to understand the regulation of importin-α isoform expression is just the tip of the iceberg; a thorough analysis of importin-α isoform expression, *in vivo* interactions and post-translational regulation will allow better understanding of their role in cell physiology. Due to their function as gatekeepers to the nucleus and unique specialization for important signalling cargoes often associated with human disease, importin-α isoforms are excellent targets for the development of new pharmacological agents.

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Abbreviations

AMPK	5′-AMP-activated protein kinase
Arm	armadillo
IBB	importin-β-binding
IBD	inflammatory bowel disease
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NLS	nuclear localization signal
NP	nucleoprotein
NPC	nuclear pore complex
nup/Nup	nucleoporin
PHB	prohibitin
PRRSV	porcine reproductive and respiratory syndrome virus
Ran	Ras-related nuclear protein
RCC	regulator of chromosome condensation
Rch1	Rag cohort 1
SNP	single nucleotide polymorphism
SRP1	serine-rich protein 1
STAT	signal transducer and activator of transcription

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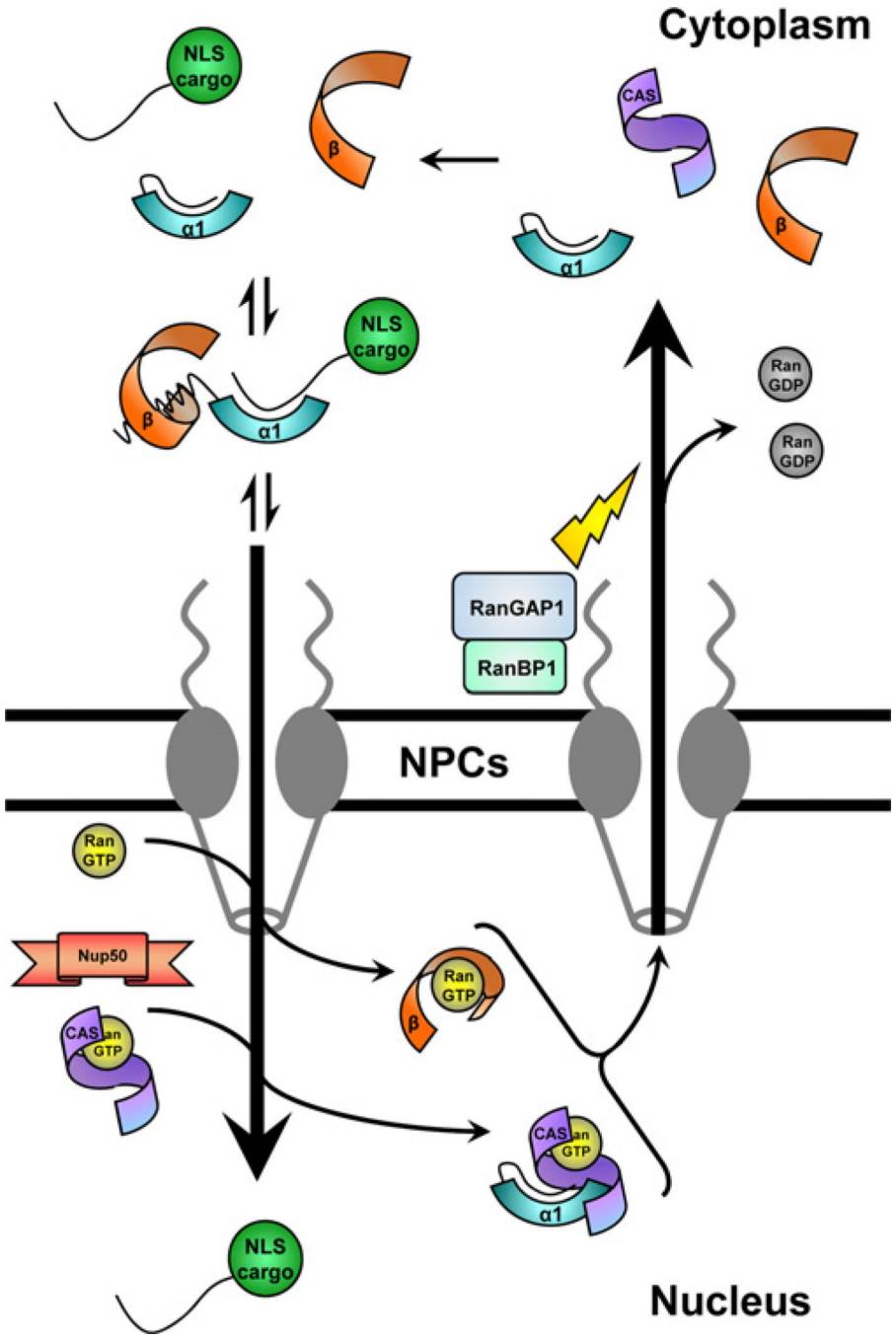

Figure 1. The classic nuclear import pathway

Importin β, importin α and an NLS-cargo assembly in the cytoplasm travel through the nuclear pore. The disassembly of the import complex is started by the binding of RanGTP to importin β, which causes release of the IBB domain. Disassembly of the NLS-cargo from importin α is a concerted effort of the IBB domain, the nucleoporin Nup50 and the importin α-recycling factor CAS. The empty importins are recycled back to the cytoplasm, where they are released to restart the import cycle via hydrolysis of RanGTP.

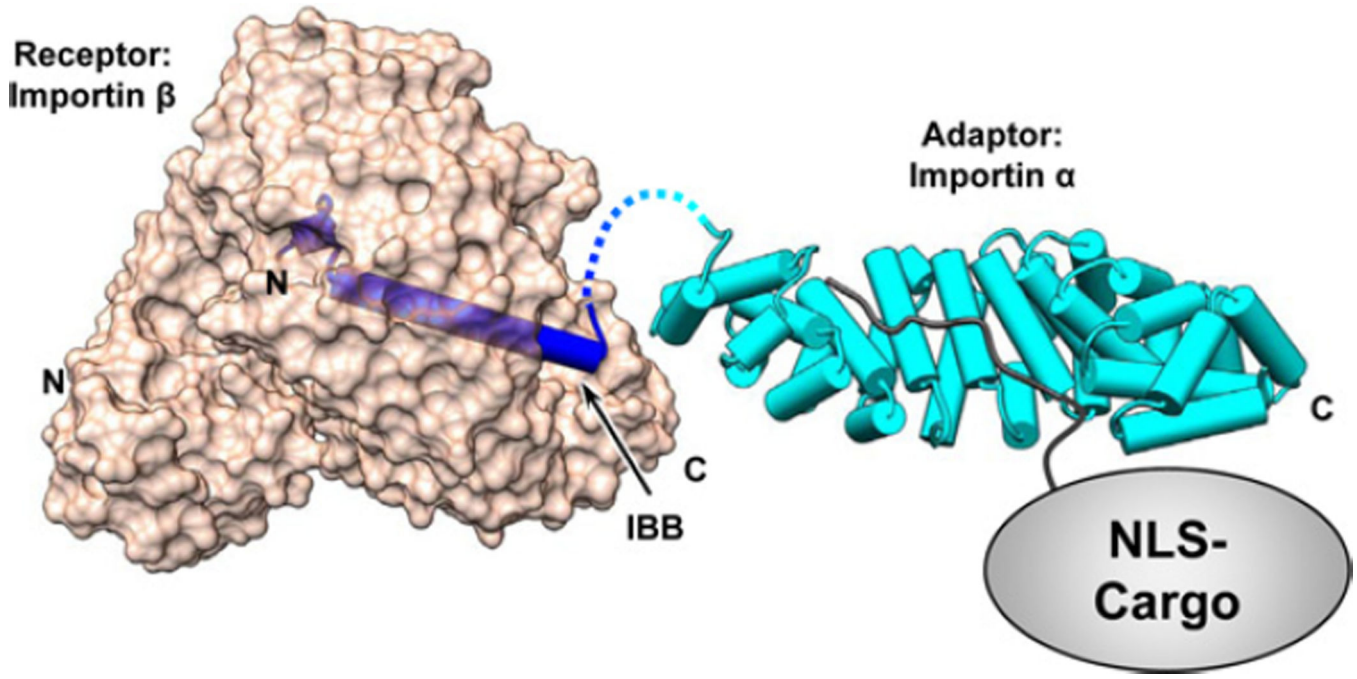

Figure 2. The classic nuclear import complex

The diagram is based on the structure of human importin β (beige) bound to the IBB domain (dark blue; PDB 1QGK) and mouse importin α2 (cyan) bound to the nucleoplasmin NLS (grey; PDB 1EJY). The N-terminus and C-terminus of each structure are noted with an 'N' or 'C', respectively. The illustration was produced using the Chimera program [150].

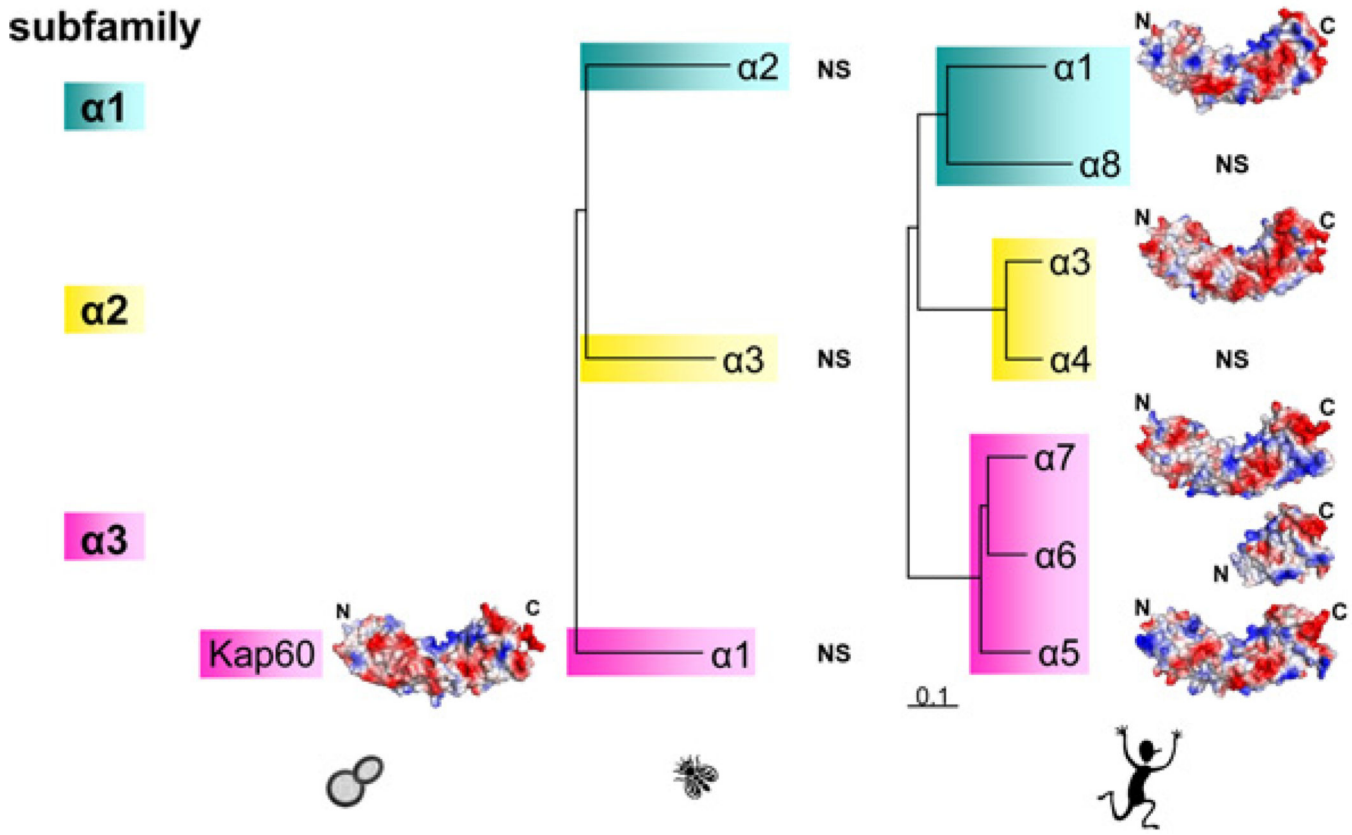

Figure 3. Evolution of importin α

From left to right is a schematic diagram of the evolution of the importin-α gene in *Saccharomyces cerevisiae*, *Drosophila* sp. and *Homo sapiens*; the scale bar represents 0.1 residue changes per amino acid position. Phylogenetic trees for *Drosophila* and human isoforms were generated with ClustalW [15] and TreeView [151]. The electrostatic surface charge distribution {determined using APBS Tools [152] and PyMol (PyMOL Molecular Graphics System, Version 1.3r1, Schrödinger L. L. C.)} is shown for the isoforms determined crystallographically, namely importin α1 (PDB 4E4 V), importin α3 (PDB 4UAE), importin α5 (PDB 3TJ3), importin α6 (PDB 4U2X) and importin α7 (PDB 4UAD). NS: Not Solved.

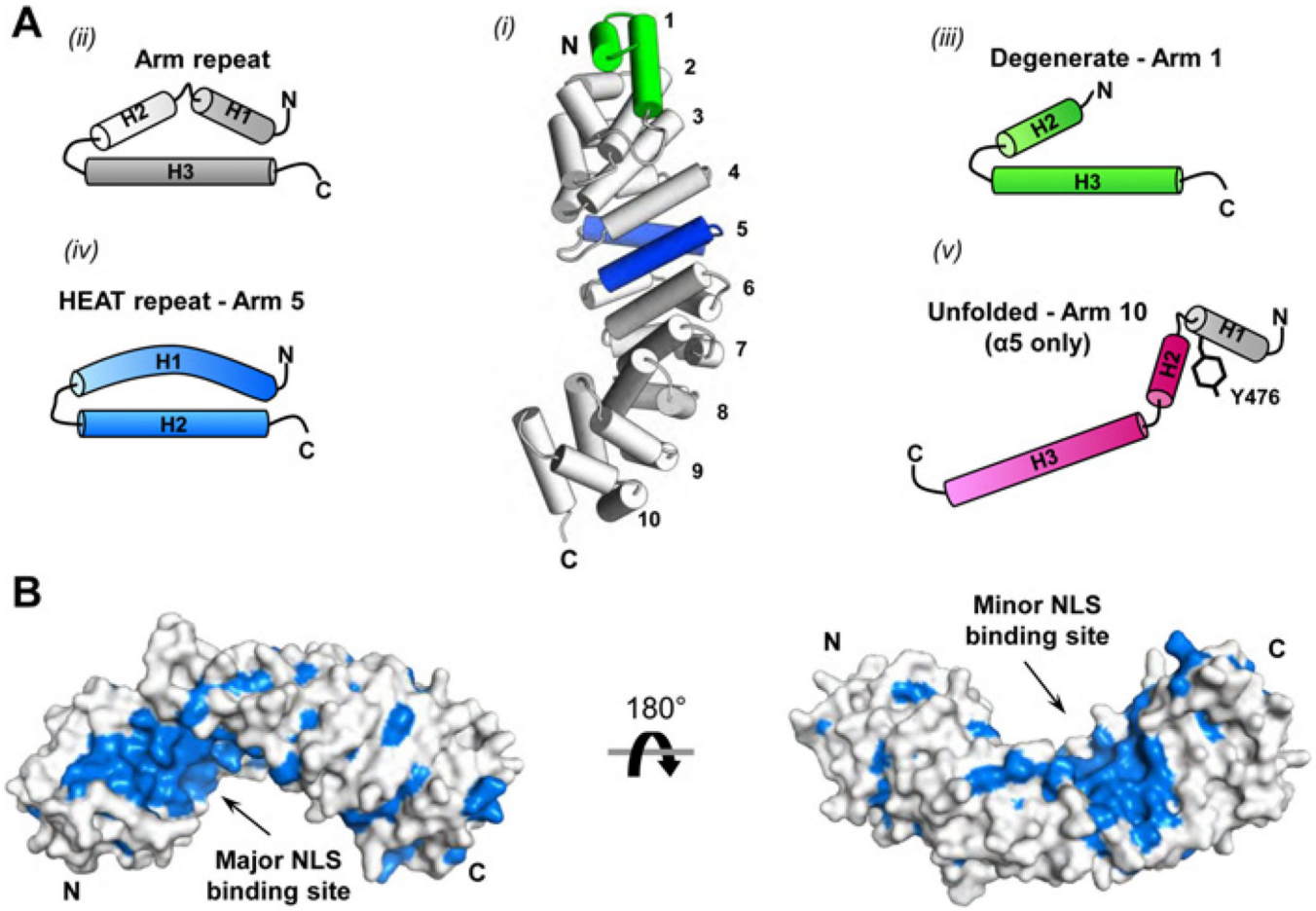

Figure 4. Arm-core structure and conservation

(**A**) (i) Structure of the Arm-core of importin α3 (PDB 4UAE) with α-helices shown as cylinders. Schematic diagram of (ii) a canonical Arm and (iii) the degenerate Arm 1, missing helix H1 and of (iv) HEAT repeat (equivalent to Arm 5), and (v) Arm 10, which adopts an open conformation in a structure of importin α5 (PDB 2JDQ). (**B**) Conservation of the NLS-binding surface of importin-α isoforms. Residues identical in all human isoforms were mapped on to a surface representation of importin α1 (PDB 4E4 V). Identical and non-conserved residues are coloured blue and grey, respectively.

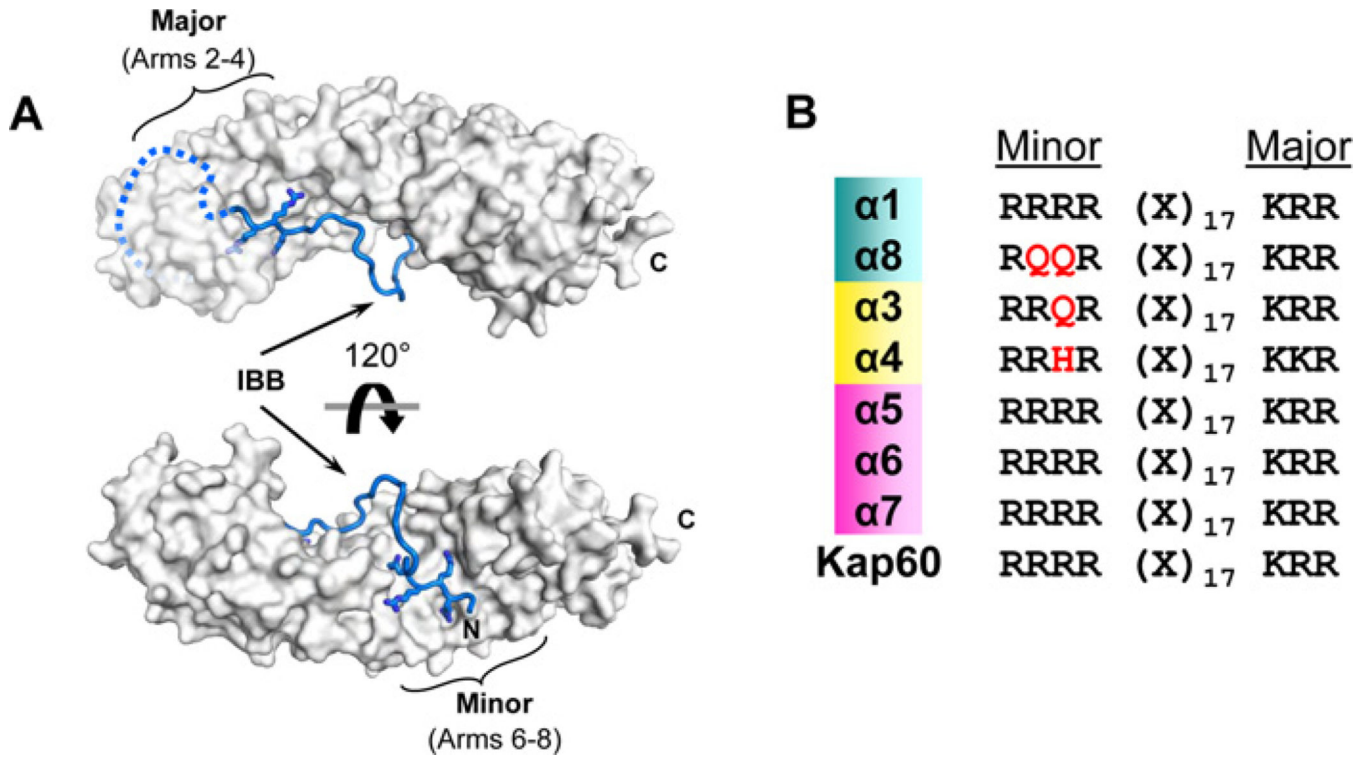

Figure 5. Autoinhibition of Arm-core by the IBB domain

(**A**) Model of the full-length importin α (grey surface) autoinhibited by the IBB domain (blue). The structure shown represents the full-length Kap60 extracted from PDB 1WA5. (**B**) Alignment of the basic residues of the IBB domain that interact with the major and minor NLS-binding sites of importin α isoforms and Kap60.

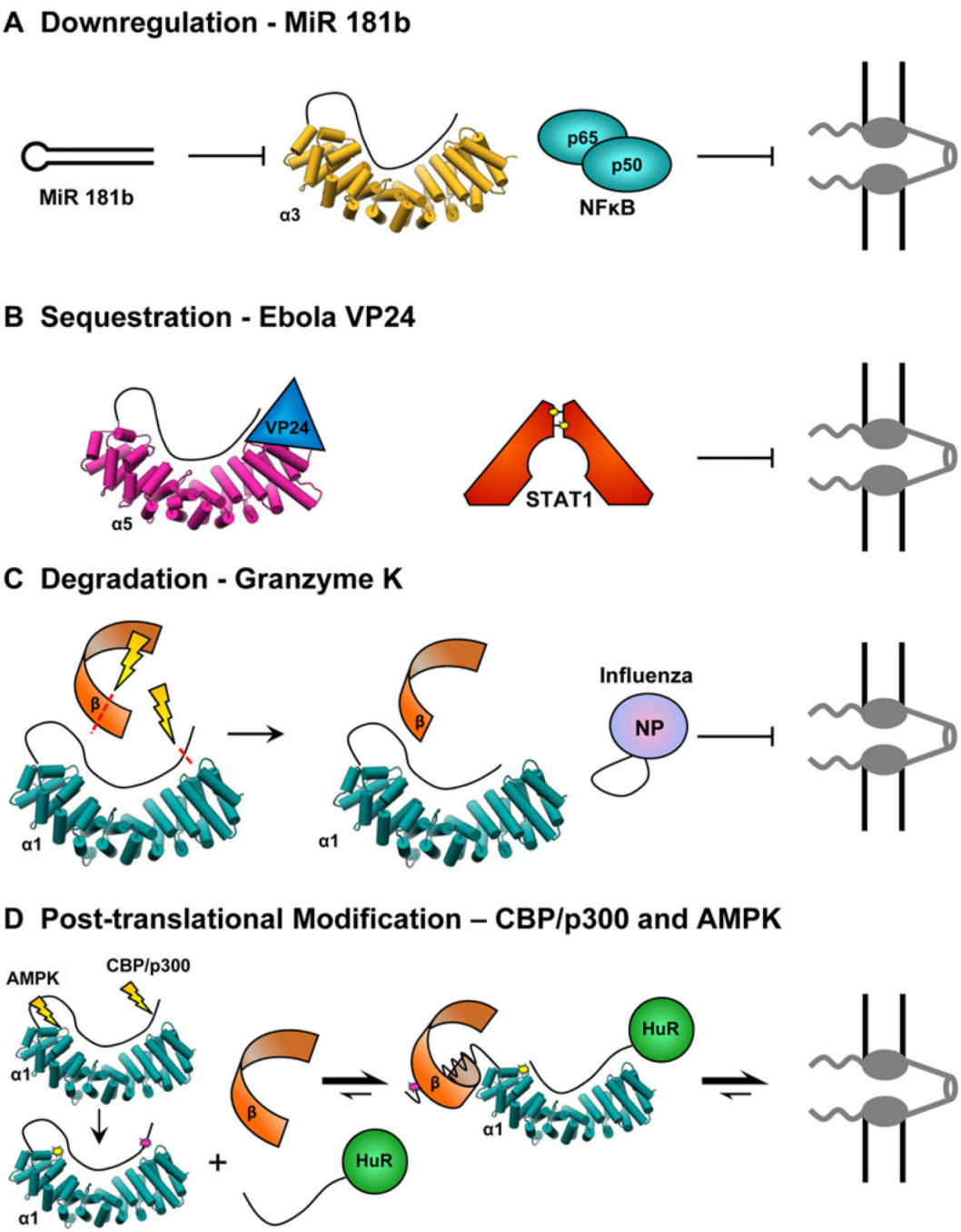

Figure 6. Cellular strategies of regulating importin-α isoforms

Schematic diagrams of: (**A**) microRNA-mediated regulation of importin-α expression; (**B**) regulation of import by sequestration of importin α; (**C**) regulation of import by degradation of importin α; and (**D**) regulation of import by post-translational modifications of importin α.

Table 1

Alternative names of importin-α isoforms

Subfamily	Protein names	Alternative names	Gene name	Accession number	PDBs
α1	Importin α1	Karyopherin α2, hSRP1α [17], Rch1 [19], Qip2 [40], NPI-3 [154]	*KPNA2*	P52292	4E4 V
	Importin α8	Karyopherin α7	*KPNA7*	A9QM74	–
α2	Importin α3	Karyopherin α4, Qip1 [40]	*KPNA4*	O00629	4UAE
	Importin α4	Karyopherin α3, hSRP1γ [155], Qip2 [156]	*KPNA3*	O00505	–
α3	Importin α5	Karyopherin α1, NPI-1 [45], SRP1 [44], hSRP1 [40]	*KPNA1*	P52294	2JDQ, 3TJ3, 4B18
	Importin α6	Karyopherin α5	*KPNA5*	O15131	4U2X
	Importin α7	Karyopherin α6, NPI-2 [157]	*KPNA6*	O60684	4UAD

Table 2

Selected cellular cargoes specific to importin-α isoforms

	Importin-α isoforms						Protein source		Experimental methods		
Cellular import cargoes	**α1**	**α3**	**α4**	**α5**	**α6**	**α7**	**α**	**c**	**Biochemical**	**Functional**	**Reference**
ADAR2	−		+ +	+ +			e	e	Y2 H	−	[158]
ADAR2R	+ +		−	−			e	e	Y2 H	−	[158]
ADAR3	+ +		−	−			e	e	Y2 H	−	[158]
AIRE	+	+ +		+ +		+	b	e	PD	−	[159]
ARHI (DIRAS3)	+ +	+ +		-	+ +	+ +	h	h	PD	−	[129]
Arx (NLS1)	−	+ +		+ +	−	−	b	h	PD	IA, SI	[160]
BRCA1	+ +						e	e	Y2 H	−	[161]
BRMS1	−	−			+ +		e	e	Y2 H	−	[162]
Brn2	−	−		+ +			b	b	−	IA	[55]
Cby	+	+ +		+ +	+ +		b	b	DI	−	[163]
CIC (mammalian capicua)	−	−	+ +	−	−	−	h	h	IP, MS	−	[164]
CREB2 (murine)	−	−	−	+ +		+ +	h	b	PD	−	[165]
Daxx	−	+ +	−	−	−	−	b	e	PD	−	[166]
DGKζ	−	+ +		+ +			b	h	PD	−	[167]
DNA Helicase Q1/RecQL	+ +	+ +		+			b	h	PD, DI	IA	[39,40]
DYRK4	−	+ +		+ +			b	h	IP	−	[168]
E47 (transcription factor)	+ +	+ +	−	−			b	b	−	IA	[169]
Endostatin	+ +	−		−		−	h	h	−	SI	[170]
FGF1							h	h	−	SI	[171]
GLUT2	+ +			−			b	e	Y2 H, IP	DN	[172]
GlyR		+ +	+ +				b	b	Y2 H, DI	−	[173]
hnRNP C1/C2	−	+ +	+ +	+ +	+ +	+ +	h	h	IP, MS	−	[174]
HuR	+ +	−	−	−		−	h	h	IP, DI	−	[141]
IRF-1	+ +						h	h	−	SI	[175]
IRF-9	−	+ +	+ +	−	−	+	e	b	PD	−	[176]
Itk	+ +			−			h	h	Y2 H, IP	−	[177]
JNK1	+ +	−	−				h	h	−	IA	[178]

	Importin-α isoforms						Protein source		Experimental methods		
Cellular import cargoes	**α1**	**α3**	**α4**	**α5**	**α6**	**α7**	**α**	**c**	**Biochemical**	**Functional**	**Reference**
Keap1	−	−	−	−	−	+ +	e	b	PD	SI	[179]
Kir/Gem	−	−	−	+ +	−	−	b	h	PD, IP	SI	[180]
LSD1	−	−	+	+ +		+ +	h	b	PD	−	[181]
Mycd (rat)	−	−		+ +			h	h	IP	−	[182]
NBS1	+ +	−		−			e	e	Y2 H	SI	[183]
NF-κB (p50/p65)	−	+ +	+ +	+ +	+ +	−	b	e	PD	−	[43,124]
NF-κB (p65)	+ +	−	−	−	−	−	h	h	−	SI	[184]
NF-κB c-Rel	−	−	−	+ +	+ +	+ +	b	e	PD	−	[124]
NF-κB p52	−	+ +	+ +	+ +	+ +	−	b	e	PD	−	[124]
NF-κB RelB	−	−	−	+ +	+ +	+ +	b	e	PD	−	[124]
Notch intracellular domain	−	+ +	+ +	−	−	+ +	b	h	PD	SI	[185]
NUCKS	−	+ +		+ +			b	h	PD	−	[186]
Oct6	−	+ +		+ +			b	b	−	IA	[55]
Orc6	−	−	−				h	h	MS	−	[187]
P27 (CDK inhibitor)	−	+ +		+ +			b	b	DI	IA	[188]
P53	−	+ +		−	−	−	b	h	PD	−	[189]
PABPC	−						b	h	PD, DI	−	[190]
Par3	−	−	+ +	−		−	h	h	MS	−	[191]
PARP-2	+	+ +		+			b	h	PD	−	[192]
Pin1	−	+ +	−	+ +		−	h	h	IP	−	[193]
PKM2/PIN1	−	−	−	+ +		−	h	h	IP	SI	[194]
RAC3	−	+ +	−	−	−	−	b	e	PD, Y2 H	−	[195]
RanBP3	−	+ +	+ +	−	−	−	b	b	DI	IA	[196]
RCC1	−	+ +	+ +	−	−	−	b	b	DI	IA	[21,197]
RNA helicase A	+ +	+ +	−	−	−	−	b	b	DI	IA	[198]
Serum response factor	+	−	−	+ +			b	e	IP, PD	IA	[199]
SET	−	+ +	+	−	−	−	b	b	IP, DI	−	[200]
pSTAT1	−			+ +			b	h	IP, PD	MI	[46]
pSTAT1 and pSTAT1/pSTAT2	−	−		+ +		−	b	h	PD	−	[81]
pSTAT1	−	−	−	+ +	+ +	+ +	h	h	IP	−	[93]

	Importin-α isoforms						Protein source		Experimental methods		
Cellular import cargoes	**α1**	**α3**	**α4**	**α5**	**α6**	**α7**	**α**	**c**	**Biochemical**	**Functional**	**Reference**
pSTAT1	−	−	−	+ +		+ +	h	h	IP, PD	−	[201]
uSTAT3/pSTAT3	−	+ +		−	+ +	−	b	h	PD	SI	[202]
pSTAT3	+	+		+ +			b	h	PD	DN	[156]
pSTAT3	−	−	−	+ +		+ +	h	h	IP, PD	−	[201]
uSTAT3/pSTAT3	+	+ +		−	+ +	+ +	b	h	PD	−	[129]
STAT5 a	−	+ +		−	+ +	−	b	h	PD	SI	[203]
STAT6	−			−		−	b	h	PD	−	[204]
TBP-2	+ +	−		−			e	e	Y2 H	SI	[205]
Tpr	+ +	−	−	+	+	+	e	b	PD	SI	[206]
Wt1	+ +	−	−	−		−	b	e	PD	−	[207]
XPA	−	−	+ +	−	−	+	h	h	IP	SI	[208]
ZIC3	−	−		+ +		+ +	b	e	PD	SI	[209]
Znf131	+	+ +		+		+	b	e	PD	−	[210]

Cargoes needed to be either tested with at least two importin-α isoforms or screened with multiple isoforms available, and from these experiments have shown some degree of specificity. Importin-α isoforms: importin α8 was not included because no cargoes specific to it have been identified yet. A blank indicates that an import cargo was not tested for binding to an importin-α isoform, (−) no interaction, (+) a weak interaction or an interaction that wasn't relevant for nuclear import, and (+ +) a strong interaction or confirmed preference from a nuclear import assay. Protein source refers to the expression system used to generate importin α (α) and cargo (c): 'b' = bacteria, 'e' = eukaryotic and specifically 'h' = human cells. Only proteins expressed in a eukaryotic expression system carry post-translation modifications.

Experimental methods indicate the assay used to determine physical interactions between an importin-α isoform and a cargo: 'IP' = immunoprecipitations, 'PD' = pull downs, 'DI' = pull downs with purified proteins to ensure physical direct interaction, 'Y2H' = yeast-two hybrids, 'MS' = mass spectrometry after IP, 'IA' = reconstituted import assays in permeabilized cells, 'SI' = knock down by siRNA, 'MI' = microinjection, 'TR' = transfection, 'DN' = dominant negative importin α5 and 'KD' = full *in vivo*knock down.

Table 3

Selected viral cargoes specific to importin-α isoforms

	α Isoforms						Protein source		Experimental methods		
Viral import cargoes	**α1**	**α3**	**α4**	**α5**	**α6**	**α7**	**α**	**c**	**Biochemical**	**Functional**	**Reference**
Adenoviral E1A	+	+ +	+	+		+	b	b	–	IA	[211]
Adenoviral E1A	+ +	+ +		+ +			h	h	IP	–	[212]
BAdV-3 5K2	–	+ +		–		–	b	e	PD	–	[213]
Chickunguya virus capsid	–	+ +	–	–			e	b	PD	–	[214]
Epstein–Barr virus pEBNA-1	–	–		+ +			b	b	DI	–	[139]
Ebola VP24	–	–	–	+ +	+ +	+ +	h	h	IP	–	[93]
Haantan virus N	+ +	+ +	–	+ +			h	h	IP	–	[215]
HIV-1 integrase	–	+ +		–		–	b	b	DI	SI	[82]
HIV-1 integrase							b	b	DI	IA	[216]
HIV-1 Vpr	+ +	+ +		+ +			b	b	PD	IA	[89]
HIV-1 Vpr	+	+		+ +			b	h	DI	IA	[90]
Human herpesvirus 6 U69	+	+	–			+ +	b	b	DI	IA	[217]
HPV E1 (bovine)	–	+ +	+ +	+ +		–	b	b	DI	IA	[218]
HPV E2	–	+ +	–	+ +		–	b	b	DI	–	[219]
Influenza – NP	+ +			+ +			e	e	Y2 H	IA	[80]
Influenza – NP	+ +	+		+ +		–	b	e	PD	–	[81]
Influenza – PB2	+ +	+	+	+		+ +	h	h	IP	SI	[85]
Influenza – PB2	–	+ +		+		+	b	b	DI	–	[35]
Influenza – PB2	–	+ +	–	–	–	+ +	h	h	–	SI, KD	[63]
Nipah virus W	–	+ +	+ +	–	–	–	h	h	IP	–	[220]
SARS-CoV ORF6	+ +	–	–	–			h	h	IP	TR	[95]
SV40 large T antigen	+	+ +	–	–	–	–	b	e	PD	–	[81]
VEE nsP2	–	–	–	+ +			h	h	IP	–	[221]

Please refer to the legend to Table 2.