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Importins and exportins in cellular differentiation

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Abstract

The importin/exportin transport system provides the machinery involved in nucleocytoplasmic transport. Alterations of the levels of importins and exportins may play crucial roles in development, differentiation and transformation. Employing human leukaemia HL-60 cells, we and others have revealed the differentiation-associated changes in the protein and gene expression of these factors. The recent finding that a switch to the importin-α subtype triggers neural differentiation of embryonic stem cells underscores the importance of nucleocytoplasmic transport factors in cellular events. This review focuses on current research into the roles of importins and exportins in cell differentiation.

Keywords: importin • exportin • differentiation • gene expression • nucleocytoplasmic transport

Introduction

In eukaryotic cells, the nucleus is separated from the cytoplasm by a double-layered membrane, the nuclear envelope. Macromolecules such as RNA transcripts generated in the nucleus are exported to the ribosomes in the cytoplasm and proteins synthesized in the cytoplasm, such as histones, DNA and RNA polymerases and transcription factors, are imported into the nucleus. The importin/exportin transport system provides the machinery involved in nucleocytoplasmic transport of cargo molecules larger than ~40 kD [1–12]. In this system, proteins that shuttle between the cytoplasm and the nucleus have generally a nuclear localization signal (NLS) sequence or a nuclear export signal (NES) sequence.

Importin-α recognizes to the classical NLS (cNLS) within a protein cargo and forms a ternary complex with importin-β_1 to enter into the nucleus (Fig. 1). In another system, the cargo molecule with the NLS directly binds to importin-β and is transported into the nucleus. Exportin recognizes the NES in the cargo protein and the complex is exported from the nucleus by binding with the guanosine triphosphate (GTP)-bound form of the guanine nucleotide-binding protein Ran (RanGTP) (Fig. 1).

Alterations in the expression of the components of the nuclear transport machinery would determine transport efficiency and plays crucial roles in development, differentiation and transformation. This review focuses on current research into the roles of importins and exportins in cellular differentiation.

Importins and exportins

The National Center for Biotechnology Information (NCBI) database shows that there are at least 18 importin and 6 exportin genes in human beings and 15 importin and 6 exportin genes in mice.

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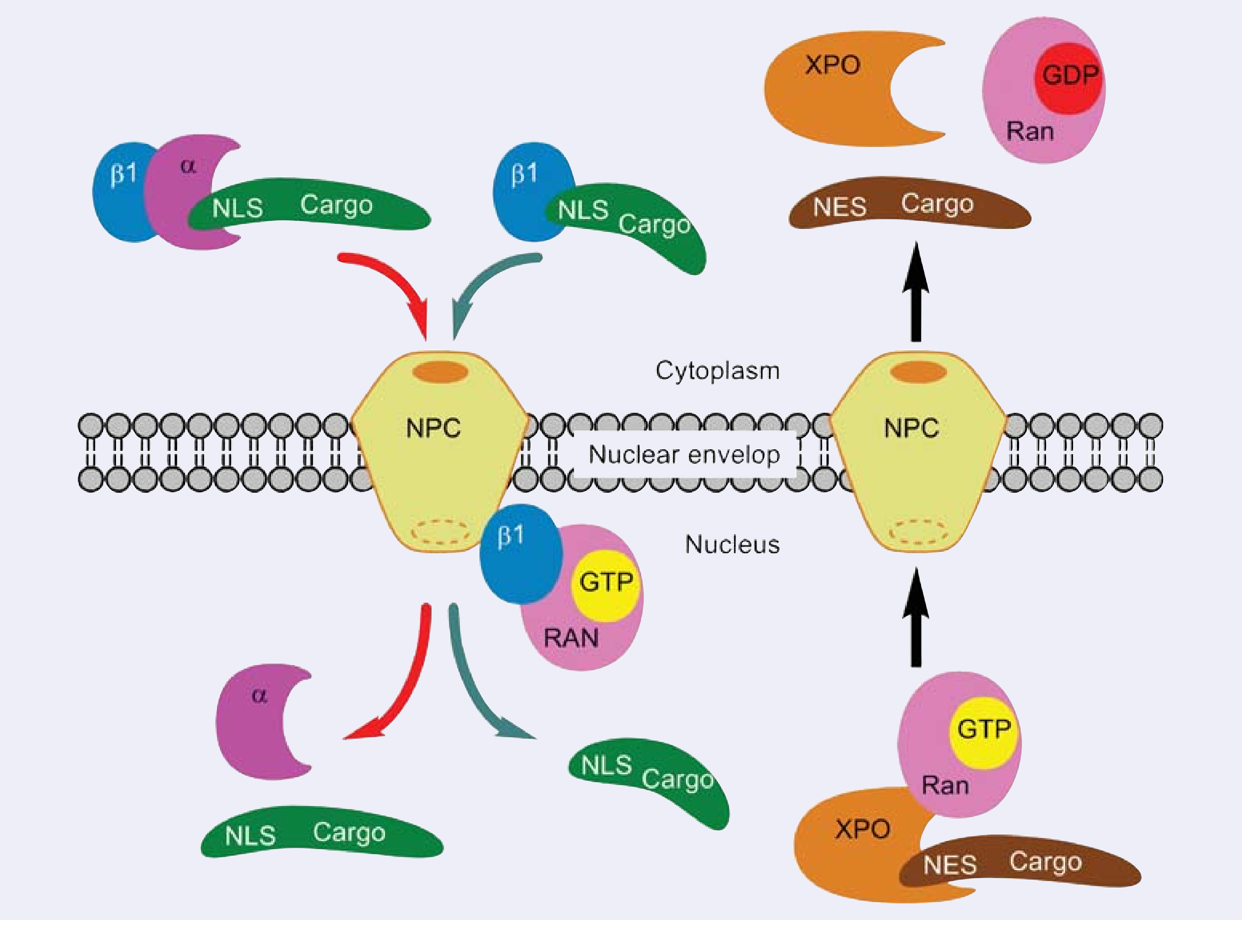

Fig.1 A basic model of importin/exportin-mediated nucleocytoplasmic transport of macromolecules. Importin-α (α) binds to the NLS within a protein cargo in the cytoplasm and forms a ternary complex with importin-β1 (β1) to enter into the nucleus. Some cargo molecules with the NLS can directly bind to importin-β1. In the nucleus, binding of RanGTP (the GTP-bound form of the small Ras family GTAse, Ran) to importin-β1 triggers the dissociation of the complex. For nuclear export, RanGTP stimulates binding of exportin (XPO) to an NES-containing cargo protein in the nucleus and the complex is exported to the cytoplasm, where hydrolysis of RanGTP to RanGDP results in complex disassembly.

Exportin-1 is frequently referred to as CRM1. Comparisons of these nucleocytoplasmic transport factors in different species have been hampered by the multiple names assigned. In this review, we use the terms for the human genes.

There have been a number of published comprehensive reports reviewing structural, functional, evolutionary, mechanistic and regulational aspects of nucleocytoplasmic transport factors including importins and exportins [1–12]. Figure 1 illustrates a basic model for nuclear import/export pathways in which importins and exportins are involved.

In the importin-α-mediated nuclear import system, cytosolic importin-β_1 forms a complex with importin-α, which binds to the cNLS contained in a cargo protein. After entering the nucleus through the nuclear pore complex (NPC), the ternary complex dissociates. The energy required for this dissociation is provided by GTP from RanGTP. Importin-α is recycled back to the cytoplasm in a complex with an importin-α re-exporter, cellular apoptosis susceptibility gene (CAS), in the presence of RanGTP [1, 9]. In some cases, importin-β_1 recognizes the cNLS within a cargo in the cytoplasm without the importin-α adapter for entrance into the nucleus. Importin-β_1 in the nucleus is recycled to the cytoplasm in a complex with RanGTP.

The nuclear export of proteins is mediated by exportins which bind to NES-containing cargo and RanGTP in the nucleus. The signal recognized by exportin-1 may be termed the classical NES. Dissociation of the ternary complex in the cytoplasm is promoted by Ran GTPase-activating protein to ensure the export of the cargo. Exportin-1 is known to be recycled into the nucleus by binding to an NPC component, Nup358 [6].

Table 1 Examples of cargo molecules transported by importins and exportins

Human transport factor	NCBI official symbol	Cargo molecule		
Importin-α_1	KPNA2	Type 1 parathyroid hormone receptor [51]	IFN regulatory factor-1[39]	Oct3/4 [20]
Importin-α_3	KPNA4	NF-κB p50/p65 [18]	RNA helicase A [52]	Oct3/4 [20]
Importin-α_4	KPNA3	NF-κB p50/p65 [18]	Bovine papillomavirus type1 E1 protein [53]	
Importin-α_5	KPNA1	Stat3 [54]	Ebola virus VP24 [55]	Oct3/4 [20]
Importin-β_1	KPNB1	Splicing factor PRPF31 [56]	Sex-determining factor SRY [57]	
Importin-β_2	TNPO1	HPV16 E6 oncoprotein [58]	HPV L1 major capsid proteins [59]	
Importin-β_3	RANBP5	c-Jun [19]	Influenza A viral ribonucleoprotein [60]	NFAT [61]
Importin-7	IPO7	c-Jun [19]	Zinc finger protein EZI [62]	Histone H1 [63]
Importin-8	IPO8	Signal recognition particle protein 19 [64]		
Importin-9	IPO9	c-Jun [19]	Protein phosphatase 2A [65]	
Importin-11	IPO11	Ribosomal protein L12 [66]	Ubiquitin-conjugating enzyme UbcM2 [67]	
Importin-13	IPO13	NF-YB/NF-YC heterodimer [68]	c-Jun [19]	Myopodin [69]
Transportin-2	TNPO2	mRNA [70]	HuR [71]	hnRNP A1 [72]
Exportin-1	XPO1	Cyclin D1 [73]	p53 [5]	Survivin [48]
Exportin-5	XPO5	Double-stranded RNA binding protein Staufen2 [74]	Pre-miRNAs [75]	
Exportin-6	XPO6	Profilin-actin complexes [76]	Actin [32]	
Exportin-7	XPO7	IFN-α_1 mRNA [77]	p50RhoGAP [78]	
Exportin-t	XPOT	Mature tRNAs [79]	tRNA-attached ribozymes [80]	

NLS and NES

NLSs are nuclear targeting sequences which are recognized by importins. The best-characterized NLSs are cNLSs that have either one (monopartite) stretch such as PKKKRKV in SV40 large T antigen and EEKRKR in NF-κB p65 [5] or two (bipartite) stretches of basic amino acids. Some cNLSs are recognized directly by importin-β_1 as exemplified by the sequence RKKRRQRRR in Hiv-1 Tat [13]. In the GenBank^{TM} set of 5850 yeast proteins, 2639 (45%) proteins contain either a predicted monopartite or bipartite cNLS, suggesting the high prevalence of the classical nuclear import pathway [9].

Non-classical NLSs bind directly to the different importin-β homologues [3]. For example, the NLS with no cluster of basic amino acids in heterogeneous nuclear ribonucleoprotein A1 and other proteins is directly recognized by importin-β_2/transportin-1/karyopherin-β_2 [14]. In addition, importin-independent nuclear entry systems are also known. These include viral protein R (Vpr) of immunodeficiency virus type 1 (HIV-1) and β-catenin, which can pass through the NPC by binding directly to NPC components [15, 16].

NESs recognized by exportins generally have short sequences with a cluster of hydrophobic amino acids such as RFLSLEPL and TPTDVRDVDI in cyclin D [5] and LQKKLEELEL in mitogen-activated protein kinase kinase [6]. Although exportin-1 has low affinity for regular NESs to achieve efficient release of export complexes from the NPC, there is another signal recognized by exportin-1 with high affinity [6]. One example is snurportin, which does not contain a canonical NES sequence but binds to exportin-1 through a larger domain [6].

Various types of intra- and inter-molecular masking of these transport signals regulate the efficiency of nucleocytoplasmic transport. Phosphorylation, changes in calcium concentrations and conformational changes for self-inhibition are representative events for such masking [5, 11].

Table 2 Selected studies on differentiation-associated changes in gene/protein expression of importins and exportins

Cells	HL-60	HL-60	HL-60	HL-60	ESC‡	ESC	Monocytes
Cell fate	**Macrophages**	**Granulocytes**	**Macrophages**	**Granulocytes**	**Cardiomyocytes**	**Neural cells**	**Macrophages**
Method	**Q-PCR**	**Q-PCR**	**WB§**	**WB**	**Q-PCR**	**WB**	**Q-PCR/WB**
Importin-α_1	Down	Down	Down	Down		Down	Up
Importin-α_3	Down	NC¶	Down	NC		Up	Up
Importin-α_5	Up	Up	NC	NC	Down	Up	Up
Importin-β_1	Down	Down			Down		
Transportin-2	Down	Down			Up		
Exportin-1	Down	Down			Down		
Exportin-5	Down	Down					
Exportin-6	NC	Down					
Exportin-7	Down	Down	Down	Down			
Exportin-t	Down	Down					
Ref.	[25]	[25]	[23]	[23]	[38]	[20]	[33]

‡Embryonic stem cells.
§Western blotting.
¶No change.

Cargo molecules

Table 1 lists examples of macromolecules transported by nucleocytoplasmic transport factors. The importins and exportins selected here are those for which information on differentiation-associated changes in gene expression is available through our cDNA microarray analysis in human promyelocytic leukaemia HL-60 cells [17]. In many cases, an individual protein is carried by a specific importin or exportin, but some proteins are recognized by multiple isoforms as exemplified by NF-κB [18], c-Jun [19] and Oct3/4 [20] (Table 1).

Importins and exportins in cellular differentiation

HL-60 cell differentiation

Importin expression

HL-60 cells can be induced to differentiate into monocyte/macrophage-like and neutrophil-/granulocyte like cells in response to external stimuli such as 1α,25-dihydroxyvitamin D3, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), *all-trans*-retinoic acid (ATRA) and dimethylsulfoxide [21, 22].

The protein expression of importins-α_1 (Table 2) and -α_4 is greatly repressed in differentiated HL-60 cells, while that of importin-α_7 is weakly down-regulated [23]. The protein expression of importin-α_3 is down-regulated upon differentiation towards macrophage-like cells in contrast to the stable expression in cells differentiating into granulocyte-like cells [23] (Table 2).

Consistent with the protein expression, the gene expression of most importins is down-regulated upon differentiation as examined by a cDNA microarray analysis [17] and a Q-PCR [24, 25] (Table 2). The result of Q-PCR indicates that the gene expression of importin-α_5 is up-regulated upon differentiation towards macrophage-like cells [25] (Table 2).

The changes in the gene expression of importin-α_3 are also compatible with those in the protein expression associated with the difference in differentiation of HL-60 cells. Thus, importin-α_3 appears to have a very important role in directing cell lineages, monocyte/macrophages *versus* neutrophil/granulocytes [23].

ATRA induces a reduction in the gene expression of importin-α_1 in HL-60 cells upon granulocytic differentiation (Table 2) with a transient up-regulation [25]. Similar observation has been made for cultured rat aortic smooth muscle cells [26].

The down-regulation of the gene expression of proteins related to nucleocytoplasmic transport may explain the differentiation-associated suppression of the growth of HL-60 cells [27, 28]. Another example of the involvement of importins in cell growth is the finding that RNAi-based down-regulation of the gene expression of importins-α_3, -α_5, -α_7 and -β_1 strongly inhibits the proliferation of HeLa cells [29].

The down-regulated expression of importin-α_1 accompanied by the up-regulated expression of importin-α_5 is seen in HL-60 cells both during TPA-mediated differentiation into macrophage-like cells and ATRA-mediated differentiation into granulocyte-like cells [25] (Table 2). The observation is in line with a recent finding that this switching triggers neural differentiation of mouse embryonic stem (ES) cells [20] (Table 2). The possibility that this switching is a hallmark of cell differentiation should be studied further.

Exportin expression

While the gene expression of five exportins (exportins-1, -5, -6, -7 and -t) is down-regulated in HL-60 cells differentiating towards granulocyte-like cells, the level of exportin-6 is maintained in HL-60 cells differentiating into macrophage-like cells [17, 24, 25] (Table 2). The difference in the expression of exportin-6 in addition to importin-α_3 may be related to the differentiation of HL-60 cells into different lineages [25].

The down-regulation of exportins may be involved in the differentiation-associated inhibition of cell growth [27, 28]. Leptomycin B, an inhibitor of exportin-1, is known to prevent proliferation and cause cell cycle arrest at both G1 and G2 in rat 3Y1 fibroblasts [30]. Ratjadones inhibiting nuclear export by blocking exportin-1 also inhibit the growth of several types of eukaryotic cells [31].

Microinjected β-actin is accumulated in the nucleus of *Xenopus* oocytes unless exportin-6 is coinjected [32]. Thus, exportin-6 specifically mediates the nuclear export of β-actin and actin isoforms, and its expression is developmentally regulated in embryogenesis. The nuclear accumulation of actin has been observed in cells treated with dimethylsulfoxide, which is an inducer of the differentiation of HL-60 cells toward granulocyte-like cells [32], and it is worth examining its possible relationship with the down-regulated gene expression of exportin-6 as observed in HL-60 cells differentiating into granulocyte-like cells with ATRA [25] (Table 2).

Monocyte differentiation

Macrophages induced to differentiate by macrophage colony-stimulating factor express higher levels of proteins and mRNAs for importins-α_1, -α_3 and -α_5 than undifferentiated monocytes from human peripheral blood [33] (Table 2). Since HIV-1 Vpr is able to use these importins for nuclear entry, the observation provides an explanation of why monocytes are refractory to HIV-1-based vector transduction unlike mature macrophages [34]. The interaction between Vpr and importins may be a potential target for an antiviral agent by inhibiting nuclear entry.

Terminal erythroid differentiation

Terminal erythroid differentiation is the process by which immature precursor cells become erythrocytes in mammals. Exportin-7 appears to be very important to this event, since its gene expression is time-dependently up-regulated by erythropoietin treatment in erythroblasts isolated from the spleens of mice infected with an anaemia-inducing strain of the Friend leukaemia virus [35]. Its precise role, however, is not clear at present.

Neural differentiation

The expression of importin-α subtypes is strictly regulated during the neural differentiation of mouse ES cells [20]. The level of importin-α_1 protein is high in undifferentiated ES cells, whereas the levels of importins-α_3 and -α_5 are low and undetectable, respectively (Table 2). The RNAi-based knockdown of importin-α_1, the overexpression of importin-α_5 or a combination thereof leads to neural differentiation. The transcription factors Oct3/4, SOX2 and Brn2 which play important roles in neural differentiation contain a single cNLS (Oct3/4 and Brn2) or two cNLSs (SOX2), and importin-α_1 is involved in the nuclear transport of Oct3/4, which has a critical role in the maintenance of an undifferentiated ES-cell state. A decrease in importin-α_1/Oct3/4 concomitant with the up-regulation of importin-α_5, which is involved in the nuclear transport of SOX and Brn2 appears to lead to neural differentiation. Thus, the coordinated regulation of importin subtypes and their transcription factors appears to have a key role in cell-fate determination.

Surprisingly, transgenic Imp-$\alpha_5^{-/-}$ mice do not exhibit any obvious morphological or behavioural abnormalities [36]. Since the expression of importin-α_4 is markedly increased in the brains of these knockout mice, a compensative mechanism may cover the lack of an importin subtype in mammals. Supporting this notion, an *in vitro* transport assay has shown that both importin-α_5 and -α_4 can import Brn2, although with differences in efficiency [37].

Cardiac differentiation

In cardiomyocytes differentiated from mouse ES cells, the gene expression of nuclear transport factors including importins, exportins, transportins, nucleoporins and Ran-related factors is globally down-regulated with a few exceptions as compared to ES cells [38]. In contrast to that during the neural differentiation of ES cells, the expression of importin-α_5 is down-regulated (Table 2), suggesting that the difference may be related to cell fate. The up-regulated gene expression of transportin-2 and Ran-binding protein 6 is noticeable and may be related to the nuclear entry of cardiac transcription factors such as Mef2C, Nkx2.5 and Gata4.

Keratinocyte differentiation

Normal human epidermal keratinocytes (NHEKs) express the genes for importins-α_1, -α_3, -α_4 and -α_5, but not importin-α_6 [39]. Stimulation with interferon (IFN)-γ, a modulator of epidermal proliferation and differentiation, up-regulates the protein expression of importin-α_1 after 24 hrs, but down-regulates it by 48 hrs in NHEKs, corresponding to the mRNA expression. IFN-γ does not affect the gene expression of other importins. Since IFN-γ induces the expression of marker genes of keratinocyte differentiation, an increased nuclear entry of importin-α_1-mediated signals at an early stage of IFN-γ treatment may facilitate the differentiation.

These observations may be related to the finding that importin-α_1 is involved in the nuclear transport of IFN regulatory factor-1, a mediator of epidermal differentiation induced by IFN-γ. Overexpression and RNAi-based knockdown experiments have identified 54 genes modulated putatively by importin-α_1 in NHEKs, including the genes for involucrin, keratin-1 and -10 [39]. However, overexpression of importin-α_1 appears to induce no morphological changes as seen in differentiated keratinocytes, suggesting that importin-α_1 by itself may not be sufficient to induce the full differentiation.

Germ cell maturation

Proteomic profiling of differentially expressed proteins in germinal vesicles and metaphase II arrested mouse oocytes has identified 12 proteins including importin-α_1 that migrated differently on electrophoresis in a two-dimensional gel [40]. Thus, post-translational modification appears to take place during the maturation of oocytes.

In spermatogenesis, the mRNA expression of individual importin-α isoforms is differentially regulated [41]. Importin-α_5 is expressed in differentiated spermatogonia through to the round spermatids in the adult mouse testis, suggesting its importance in mitotic and meiotic germ cells. The expression of importin-α_1 is very limited, as its mRNA is present in spermatocytes but absent once the spermatids begins to elongate. Importin-α_4 is expressed specifically in the mitotic germ cell populations, and importin-α_3 in pachytene spermatocytes. Thus, mammalian spermatogenesis appears to be a model useful for further examination of the roles and regulation of nucleocytoplasmic transport factors in cellular differentiation and development, and information derived therefrom may have relevance to reproductive medicine.

The cellular and subcellular distribution of importins in spermatogenesis has been demonstrated comprehensively [42–44].

Muscle cell differentiation

The mouse muscle myoblast cell line C2C12 provides an excellent model for studying myogenesis *in vitro* and cell differentiation. The RNA-binding protein HuR is critically involved in the formation of muscle fibres through its association with MyoD and myogenin mRNAs and is transported into the nucleus through a transportin-2-mediated pathway [45]. Transportin-2 is expressed in undifferentiated and differentiated C2C12 cells and transportin 1 appears to be expressed weakly only in mature myotubes. The involvement of transportin-2 in muscle cell differentiation has been demonstrated by an experiment in which RNAi-mediated depletion of transportin-2 expression lead to the expression of the myogenic transcription factors MyoD and myogenin. The disruption of the association between HuR and transportin-2 appears to be an important event leading to muscle cell differentiation.

Concluding remarks

Significant progress has been achieved in our understanding of the structure and function of nucleocytoplasmic transport factors including importins and exportins. Yet, information on which specific transport factors are expressed in which tissues and cells is still limited. Such information will be crucial to investigations aiming at human therapeutic applications. In addition, further studies should be done to see whether the results obtained from *in vitro* culture models will hold true *in vivo* as well.

Many trials are in progress as exemplified by the coupling of NLS peptides to DNA for gene therapy [11] and disruption of the interaction of NF-κB with importins-α_1 and -α_5 by NLS peptides [46]. Importin-α_1 may have prognostic value in cancer [47], and inhibitors selectively targeting the survivin–exportin-1 interaction may be of therapeutic relevance [48].

Recently, the genes for transcription factors Oct4 and SOX2 have been identified as the minimum requirement for the reprogramming of human somatic cells to pluripotency [49]. Oct4 can be transported into the nucleus in a complex with either importin-α_1/-β_1, importin-α_3/-β_1 or importin-α_5/-β_1 [20]. SOX2 may be imported with either of importin-α_3/importin-β_1, importin-α_5/-β_1 or importin-β_1 [20]. The importin-β_1 gene is one of the genes downstream of Oct4 [50]. Thus, nucleocytoplasmic transport factors are a potential target also in the field of regenerative medicine.

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